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FUNCTIONAL ANALYSIS OF VIRULENCE IN VIBRIO CORALLIILYTICUS RE22 AND PROBIOTIC MECHANISMS OF PHAEOBACTER INHIBENS S4

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FUNCTIONAL ANALYSIS OF VIRULENCE IN VIBRIO CORALLIILYTICUS RE22 AND PROBIOTIC MECHANISMS OF PHAEOBACTER INHIBENS S4

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Abstract

Infections by pathogenic marine bacteria present a major problem for both the shellfish and finfish aquaculture industries, resulting in severe disease and high mortality. Pathogen infection seriously affect aquaculture production, and cause significant economic loss. Marine pathogens like *Vibrio coralliilyticus* frequently cause disease in a variety of shellfish. The use of antibiotics in large-scale aquaculture settings leads to the development, and potential transfer, of antibiotic resistance. In order to mitigate this emerging threat, an understanding of pathogenic mechanisms of infection and novel preventative strategies, such as probiotic treatment, is paramount for understanding and preventing future disease.

In manuscript I, "Two Type VI Secretion Systems in *Vibrio coralliilyticus* RE22Sm exhibit differential target specificity for bacteria prey and oyster larvae", the antibacterial and anti-eukaryotic roles of the two T6SSs (T6SS1 and T6SS2) against *E. coli* Sm10 cells and *Crassostrea virginica* larvae were evaluated. Mutations in *hcp* and *vgrG* genes were created and characterized for their effects upon bacterial antagonism and eukaryotic host virulence. Mutations in *hcp1* and *hcp2* resulted in significantly reduced antagonism against *E. coli* Sm10, with the *hcp2* mutation demonstrating the greater impact. In contrast, mutations in *vgrG1* or *vgrG2* had little effect on *E. coli* killing. In eastern oyster larval challenge assays, T6SS1 mutations in either *hcp1* or *vgrG1* dramatically attenuated virulence against *C. virginica* larvae. Strains with restored wild type *hcp* or *vgrG* genes reestablished T6SS-mediated killing to that of

wild type *V. coralliilyticus* RE22Sm. These findings suggest that the T6SS1 of *V. coralliilyticus* RE22Sm principally targets eukaryotes and secondarily bacteria, while the T6SS2 targets bacterial to a large extent than larval oysters. Attenuation of pathogenicity was observed in all T6SS mutants, demonstrating the requirement for proper assembly of the T6SS systems to maintain maximal virulence in either system.

In manuscript II, "The Role of Quorum Sensing in *V. coralliilyticus* RE22Sm during oyster infection", the pathogenic contributions of four quorum sensing (QS) genes were characterized with regard to growth ability, biofilm formation, extracellular zinc metalloprotease activity, T6SS killing ability and virulence in eastern oyster larvae. A dual function histidine kinase/phosphatase (*luxN*) mutant, a phosphorelay (*luxO*) mutant, a quorum sensing transcriptional regulator (*vcpR*) mutant, and an AHL synthase (*luxM*) mutant were created. Based on their contributions to the QS system, the *luxN*, *vcpR* and *luxM* mutants were designed to mimic a low cell density (LCD) environment, and the *luxO* mutant to mimic a high cell density (HCD) environment. Growth under shaking conditions was reduced for the *luxN*, *vcpR*, and *luxM* mutants, while growth increased in the *luxO* mutant strain. Planktonic growth reduced in the *luxO* and *luxM* mutant strains, and remained unaltered in *luxN* and *vcpR* mutant strains. Biofilm formation was increased in the *luxO* strain, and remained unaffected in *luxN*, *vcpR*, and *luxM* mutant strains. Extracellular metalloprotease production was significantly reduced in the *luxN*, *vcpR*, and *luxM* strains, and increased in the *luxO* mutant strain. Contact mediated T6SS killing was significantly attenuated in the *luxN* strain, partially attenuated in the *luxO* and *vcpR* strains, and was unaffected in the *luxM* mutant. The *luxN*, *vcpR*, and *luxM* mutants were significantly

attenuated in their ability to kill larval eastern oysters, while the *luxO* mutant strain had no effect on virulence. These data suggest an LCD state in RE22Sm attenuates virulence against larval oysters, whereas a HCD state results in wild-type levels of virulence. These data indicate that QS mediated protease activity is a secondary virulence factor in oyster infection, where the RE22Sm T6SS-1 acts as the primary virulence factor.

In manuscript III, "The Role of Quorum Sensing in *Phaeobacter inhibens* S4Sm and Effects on Probiotic Activity", the roles of four quorum sensing (QS) genes were evaluated for their effect on *N*-acyl homoserine lactone (AHL) production, cell growth, biofilm formation, inhibition of the pathogen *Vibrio coralliilyticus* RE22Sm, resilience against T6SS contact mediated attack, and protective effects on eastern oyster larvae to infection by RE22Sm. Mutations in *pgaI* (AHL synthase), *pgaR* (cognate AHL receptor and transcriptional regulator), *luxO* (phosphorelay protein), and *pgaK* (transmembrane histidine kinase/phosphate) were generated. Mutation of *pgaI* or *pgaR* resulted in overall loss of probiotic activity marked by reduced biofilm production, inability to inhibit growth of *Vibrio* sp. on agar plates, and increased susceptibility to T6SS mediated attack by *V. coralliilyticus* RE22Sm. The pretreatment of Eastern oyster larvae with the *pgaI* or *pgaR* strains resulted in partial reduction in protection as compared to wild type. The *pgaI* and *pgaR* strains were notably deficient in tropodithietic acid (TDA) production, as they lacked the characteristic yellow pigmentation the wild-type strain. Mutation of either *luxO* or *pgaK* resulted in increased by AHL production/detection. Targeted exploitation of the QS system by mutagenesis

increased the ability of S4Sm to protect larval oysters to challenge with *V. coralliilyticus* RE22Sm.

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PREFACE

This dissertation has been prepared in the Manuscript Format according to the guidelines of the Graduate School of the University of Rhode Island. The dissertation includes an introduction and the following three manuscripts:

The first manuscript: "Two Type VI Secretion Systems in *Vibrio coralliilyticus* RE22Sm exhibit differential target specificity for bacteria prey and oyster larvae" was submitted to mSphere in 2021.

The second manuscript: "The Role of Quorum Sensing in *V. coralliilyticus* RE22Sm during oyster infection" will be submitted to Applied & Environmental Microbiology in 2021.

The third manuscript: "The Role of Quorum Sensing in *Phaeobacter inhibens* S4Sm and Effects on Probiotic Activity" will be submitted to Applied & Environmental Microbiology in 2021.

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LITERATURE REVIEW

Overview of *Phaeobacter inhibens*

Abstract

Phaeobacter inhibens, a member of the Gram-negative α-Proteobacteria belongs to the *Roseobacter* clade. The *Roseobacter* clade is ubiquitous in the marine environment and plays a critical role in marine sulfur cycling. Genomic findings indicate metabolic versatility, as *P. inhibens* is capable of producing a variety of secondary metabolites, including tropodiethetic acid (TDA), a broad spectrum ionophoric antibiotic, as well as *N*-acyl homoserine lactones (AHLs). AHLs have been shown to be involved in Gram-negative bacterial quorum sensing. Additional genes for roseobacticides and siderophores can be found in the *P. inhibens* genome. When comparing the *P. inhibens* genome against other *Roseobacter* species, features novel to *P. inhibens* were elucidated. Taken together, *P. inhibens* has been demonstrated to exhibit probiotic activity with many marine hosts. TDA biosynthesis, AHL production, and biofilm formation have been shown to play an important role in the probiotic ability of *P. inhibens*.

Introduction

Roseobacter gallaeciensis was first reported in 1998 (1), and reclassified as a new genus, *Phaeobacter*, as *P. gallaeciensis* in 2006 (2). *P. inhibens* represents a closely related, yet novel, species to *P. gallaeciensis* (2). *P. inhibens* strains are typically isolated from a variety of niches in marine environments, including algae, mature bivalves, or larval cultures of marine fish (3). *P. inhibens* cell morphology has been previously described as a short, motile rod, with 1-2 flagella at one, or both, poles (3–5). Rosette formation is a hallmark of *P. inhibens* in mature cultures. Robust biofilm formation, and excellent colonization ability of marine surface environments is common in *P. inhibens* and related *Roseobacter* (4, 5). *P. inhibens* has been utilized as a probiotic treatment to reduce the density of *V. anguillarum*, a fish pathogen, to mitigate vibriosis is cod (6), or turbot larvae (7). As a result of the described ecological and aquacultural significance, this review will highlight the current research progress of *P. inhibens*, focusing on genomic findings, secondary metabolite production and the current understanding of probiotic mechanisms.

Main body

1. Secondary metabolites of *P. inhibens*

Genomic analysis of *P. inhibens* DSM 17935 indicates the presence of genes involved in novel secondary metabolites (8). Tropodithietic acid (TDA), a broad-spectrum ionophore antibiotic, inhibits many human and marine pathogens, especially *Vibrio* species (3, 9). Due to the chemical structure of TDA, the proposed mechanism of action is to disrupt the proton gradient across the cell membrane,

preventing the proton motive force from generating ATP (10). TDA production has been demonstrated to function as a dominant force for probiotic activity (4, 6). Additional secondary metabolites have been detected, like *N*-acyl homoserine lactones (AHLs), which can be produced by *Phaeobacter* and other *Roseobacter* strains. To date, one AHL synthase has been present in the genome of *P. inhibens* DSM 17395 (11). In a related *Phaeobacter* species, *P. gallaeciensis* BS107, the genome contains a hybrid polyketide synthase – non-ribosomal peptide synthase cluster. This cluster may encode enzymes involved in synthesizing pharmaceutically important natural products (12). It was also reported in *P. inhibens* BS107 that the production of potent, yet selective, algicides (roseobacticides) that modulate the symbiotic relationship between algae and bacteria upon detection of p-coumaric acid, a small molecule generated by *Emiliana huxleyi* (13, 14).

Analysis of the *P. inhibens* genome revealed siderophore production capabilities (15), in order to chelate and dissolve precipitated iron to allow for microbial metabolic involvement. Here, siderophore production allows for organisms to compete with pathogenic bacteria for iron, and ultimately outcompete organisms requiring iron for growth. This is particularly advantageous in iron-limited open ocean environments (16, 17).

In addition, *Phaeobacter inhibens* and *P. gallaeciencsis* strains encode for iron-chelating siderophores, located on one of their plasmids (15). The production of these secondary metabolites suggests the ability and potential for diverse interaction between *P. inhibens* and other marine species, and adaptation of these strains to fill specific ecological niches (18).

2. TDA production in *P. inhibens*

TDA is a sulfur containing broad-spectrum antibiotic with a broad range of inhibitory capabilities. TDA functions to inhibit a wide range of human and marine pathogens, both gram-negative and gram-positive (8), and is produced by some members of the *Roseobacter* clade (14). The structure of this molecule has been resolved (8) (Fig. 1). Through transposon mutagenesis, and screening for mutants producing less or no yellow pigment from TDA biosynthesis, 26 genes were identified to be crucial for TDA synthesis (15). These genes, in part, exist on a plasmid, including the TDA operon composed of the *tdaABCEF* gene cluster (8). The remaining involved genes are scattered throughout the genome, and are implicated in various pathways involved in primary metabolic processes.

TDA is a well-known, broad-spectrum antibiotic against human and marine pathogens, that functions as an electroneutral proton antiporter (Fig. 1). Interestingly, a previous study reported that single exposures to TDA administered via different methods, screening for resistant mutants, or prolonged exposure to incremental concentrations of TDA failed to produce resistant or tolerant strains of *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus* (19). By these reports, TDA appears to be a promising antimicrobial in the age of antibiotic resistance.

Several regulatory genes controlling TDA biosynthesis have been identified in DSM 17395. These include *tdaA* (11), *clpX* (20), *pgaI*, and *pgaR* (11, 21, 22). TdaA was shown to induce the expression of *tdaBEF* within the TDA biosynthetic operon (11). ClpX is a AAA+ ATPase chaperone for the ClpP (an ATP dependent protease),

which coordinates the formation of the ClpXP protease complex (23). PgaI and PgaR are part of the LuxIR type quorum sensing system found in *P. inhibens* (Fig. 2). These two proteins work together to regulate the AI-1/AHL-mediated QS system to up-regulated *tdaA* transcription. The loss of either *pgaI* or *pgaR* causes reduction in TDA synthesis (11). These findings indicate that QS and AHL production, and subsequent detection, is involved in TDA production (24). It has been reported that TDA also functions as an auto inducer, as supplementation of exogenous TDA into QS mutant backgrounds increased expression of TDA synthesis genes in both *P. gallaeciensis* (11) and, the related *Roseobacter*, *Silicibacter sp.* TM1040 (25).

Culture conditions affect TDA production. The strain DSM 17395 (15) produced 10-fold higher amounts of TDA when grown under shaking conditions versus static culture conditions (11). In order to adapt, detect, integrate and respond to a variety of environmental conditions and physiological signals, a complex regulatory system would be required by *P. inhibens* (11). Currently, the body of scientific literature has yet to elucidate the multifaceted and nuanced regulatory network controlling global TDA production.

3. AHL production in *P. inhibens*

Quorum sensing is a density dependent inter- and intraspecies chemical communicatory network utilized by bacteria to control numerous biological functions through the production and detection of small molecules. These molecules interact with target cells to regulate gene expression within certain bacterial species (26, 27). The most common intercellular communication class of molecules among

gram-negative organisms are the auto-inducer class I (AI-1) *N*-acyl homoserine lactones (AHL). In *P. inhibens* AHLs are synthesized by PgaI, a LuxI type synthase. The AHLs will bind, either directly or indirectly, to the cognate AHL receptor and transcriptional regulator, PgaR, which is a LuxR homolog (28, 29). The QS cascade in *P. inhibens* S4 resembles that of *Vibrio fischeri* (21). In *P. inhibens* PgaR is the QS regulator and PgaI is responsible for the synthesis of three AHLs: *N*-3-hydroxydecanoyl homoserine lactone (HSL), N-dodecanoyl-2,5-diene HSL, and *N*-3-hydroxytetradecanoyl-7-ene HSL (30). Zhao et al. (30) demonstrated that incubation with any of these three individual AHLs or any combination of them repressed transcription of virulence genes *vcpB* and *vcpR* in *Vibrio coralliilyticus.*

4. Aquaculture applications of *P. inhibens*

The use of probiotic organisms in an aquaculture setting has been proposed to have multiple modes of action and mitigates the dependence on reactive antibiotic treatments. Probionts compete with pathogenic organisms for colonization niches, compete for nutrients, chemical signals, improve host health, enhance host immune responses and improve water quality (16). While *P. inhibens* and related *Phaeobacter* species, as well as members of the *Roseobacter* clade, have been shown to exhibit probiotic activity against many marine microorganisms (6, 7), the underlying mechanism(s) of this nuanced probiotic activity have yet to be fully investigated. TDA production and probiotic involvement in *P. inhibens* has been studied and found to be essential to *P. inhibens* acting as a probiont (5, 31, 32). TDA has been demonstrated to inhibit *Vibrio* species, *Bacillus subtilis*, and *Salmonella typhimurium* (18, 19, 33, 34).

A TDA deficient phenotype was shown to lack protective ability in cod larvae against *Vibrio anguillarum* (6, 9), and eastern oyster larvae (35).

LITERATURE REVIEW

Overview of *Vibrio coralliilyticus*

Abstract

Vibrio coralliilyticus is a member of the γ-proteobacteria and a member of the genus *Vibrio* within the Vibrionaceae family (36). *V. coralliilyticus* and related *Vibrio* sp. are near ubiquitous in the marine environment. The distribution area of infectious species is enlarging with increasing ocean temperatures, and pose a significant threat to coral, both temperate and tropical (37, 38), and a broad range of bivalves (4). Like many *Vibrio* pathogens, the *V. coralliilyticus* genome encodes for a multitude of virulence factors (39, 40) including extracellular metalloproteases (41), hemolysins (42), cytolysins (39), and elements implicated in multiple secretion systems (39, 43, 44). A broad range of marine target organisms, multiple mechanisms of pathogenic activity, and increasing ocean temperatures act together to highlight *V. coralliilyticus* as a re-emerging, and efficient, pathogen. In order to combat this organism, a better understanding of the underlying mechanisms of pathogenicity is paramount. This review of the scientific literature will discuss the current knowledge of the quorum sensing system, and the type six secretion system (T6SS) in *V. coralliilyticus* RE22.

Introduction

Vibrio tubiashii was first reported by Tubiash et al. (45) in 1965 as a the causative agent of bacillary necrosis, a disease of larval and juvenile bivalve mollusks. Years later, after incorrect classification of the strain *V. tubiashii* RE98, a whole genome sequence by Richards et al. (46) in 2014 clarified this designation, and some members of *V. tubiashii* were reclassified as *V. coralliilyticus* such as strains RE98 and RE22. *V. coralliilyticus* is a pathogen of corals and larval shellfish, and is associated with coral bleaching (47, 48), worldwide reef loss, and infection of a variety of shellfish larvae including the eastern oyster (*Crassostrea virginica*) (49), and the Pacific oyster (*C. gigas*) (50, 51). *V. coralliilyticus* RE22 is a naturally occurring marine bacterium, and is particularly problematic in aquaculture, as *V. coralliilyticus* disease outbreaks rapidly cause near, or complete loss, of larvae mediated by a suite of virulence factors. Currently, two proteases, VcpA and VcpB, and one hemolysin, VchA have been characterized in this organism (39, 41, 42, 52, 53). Genomic data indicates the presence of additional virulence factors in *V. coralliilyticus* (39, 40). This literature review will discuss the current state of known aquaculture pertaining virulence factors and pathogenic mechanisms of *V. coralliilyticus* RE22.

Main Body

Pathogenic potential of *V. coralliilyticus*: Involved virulence factors

1. AI-1 quorum sensing in *V. coralliilyticus*

Quorum sensing (QS) is a density dependent system by which bacteria use small molecules, autoinducers (AI), to communicate within their local environment (54). Genes are subsequently expressed or repressed based on the presence of these auto inducer signals. *V. coralliilyticus* contains QS pathways for intraspecies communication, through an AI-1 system (55), and interspecies communication, mediated by AI-2 molecules (56). This QS pathway closely resembles the QS system in the bioluminescent *Vibrio harveyi.* The AI-1 pathway is mediated by acylated homoserine lactones (AHLs), while the autoinducer-2 (AI-2) pathway is mediated by furanosyl borate diesters. Autoinducer molecules are detected by membrane bound receptors on the cell surface by a two-component receptor protein with histidine kinase/phosphatase activity (LuxN). This receptor protein (LuxN) feeds into a common phosphorylation/dephosphorylation signal transduction pathway culminating at the LuxO protein (57). Phosphorylated LuxO indirectly inhibits the transcriptional regulator protein VcpR, a LuxR homologue found in *V. harveyi*, through the activity of five small regulatory RNAs (58). These quorum regulatory sRNAs (Qrrs) repress LuxR translation, and activate the translation of AphA. AhpA subsequently feeds back into the QS system to repress *qrr* and *luxR* expression. The AI-1 QS system controls many genes, directly and indirectly. In *V. harveyi*, LuxR controls 625 genes, AphA controls 167 genes, and 77 of these genes are coregulated by LuxR and AphA (59–61).

In *V. coralliilyticus* RE22, the transcription of two primary extracellular zinc metalloprotease genes, *vcpA* & *vcpB*, are controlled by *vcpR*. VcpA in RE22 has close evolutionary similarities to the EmpA protease found in *V. anguillarum* (39, 62).

Extracellular metalloprotease activity was suggested to be the primary virulence factor (41, 52). Kimes et al (43) demonstrated this proteolytic activity was increased in warmer temperatures. VcpA homolog, VtpR, directly inhibits expression of the hemolysin VthA (42). In *V. coralliilyticus*, the exact genes, or operon, under the control of VcpR is yet to be fully determined, as *V. coralliilyticus* utilizes shared regulatory components to discriminate between multiple autoinducers (61).

2. Secretion systems in *V. coralliilyticus*

Genomic analysis of *V. coralliilyticus* strain *Vc450* revealed the presence of type one secretion (T1SS), type two secretion (T2SS), type three secretion (T3SS), type four secretion (T4SS), and type six secretion (T6SS) system genes (39, 40, 43, 46). These virulence factor delivery systems are present in many pathogenic *Vibrio* sp. (39). Here, a review of the T6SS, its structural components, and virulence factor-associated effectors, will be described in related *Vibrio* pathogens.

3. The type six secretion system in *V. coralliilyticus*

The T6SS in *V. coralliilyticus* resembles an inverted T4 bacteriophage; first discovered in *V. cholerae* (63, 64). The T6SS primarily functions as a contact dependent effector delivery, and communicatory, translocation system (65, 66), and delivers multiple, diverse effectors directly into target cells using a dynamic firing system related to the action of contractile bacteriophage tails (67). The T6SS consists of several distinct structures constructed from thirteen conserved proteins (Fig. 3). Major structural components of the T6SS consist of an inner membrane bound

baseplate complex, a hollow, rapidly polymerizing, needle-like structure of stacked hexameric hemolysin co-regulated (Hcp) proteins, a VipA/VipB heterodimer contractile sheath encompassing the Hcp barrel, and a valine-glycine repeat protein G (VgrG). VgrG may associate with a small tetrameric amino acid, proline-alanine-alanine-arginine (PAAR), to improve puncturing ability of both bacterial and eukaryotic prey cell membranes (68, 69). Both the Hcp and baseplate complex appear essential to T6SS assembly and translocation. The baseplate complex, designated, TssEFGK-VgrG, anchors to the inner membrane of the firing cell, and is necessary for proper T6SS translocation (70). T6SS effectors are translocated, passively diffused, or bound to translocated Hcp proteins (71–73). The VgrG protein atop the Hcp barrel may be decorated with a PAAR motif to enhance prey specificity (74). In *V. cholerae,* VgrG has been demonstrated to exert actin crosslinking activity (75).

Two effector families have been characterized: peptidoglycan hydrolases (76), and phospholipases (77). Additional effector activities, such as nucleases (78), actin cross-linking (79), ADP-ribosylation (80), and pore formation (81) have been reported in *V. parahaemolyticus.* T6SS effectors are often paired with a cognate immunity protein, encoded downstream of the effector gene, to prevent self-intoxication (76, 82). The presence of T6SS associated effectors can be detected via MIX (markers of type six) effector motifs (83). Salomon et al. (83) indicated that that MIX motifs may predict T6SS mediated virulence, these motifs alone are not required for T6SS activity. Further amino acid analysis of MIX motifs indicated considerable diversity, and demonstrated the presence of a widely conserved central motif, hRxGhhYhh (where h

represents hydrophobic residues), and two less conserved motifs at the N terminus (shhPhR) and the C terminus (hhF/YSxxxWS/T). The majority of MIX sequences include extended C termini lacking identifiable relationships to known domains. Known MIX domains include the peptidoglycan binding domain LysM, PyocinS and bacteriocidal colicin DNase domains, RNase ribosomal inactivating domain (cytotoxic), and Rho activated domain of cytotoxic necrotizing factor (CNF1). MIX motifs can also include domains of unknown function (DUF), and are commonly found fused to VgrG or PAAR containing proteins, and supports the linkage of MIX motifs with T6SS (84).

T6SS MIX-effectors carry both anti-bacterial and anti-host (eukaryotic) functionality. Secretome analysis of *V. proteolyticus* indicated the presence of three T6SSs, and the T6SS1 was linked to anti-bacterial activity, and enhanced under warmer conditions (85). Against *E. coli*, inactivation of T6SS1 by (Δ*vgrG1*) resulted in complete loss of anti-bacterial activity, whereas inactivation of T6SS2 (Δ*vgrG2*) or T6SS3 (Δ*vgrG3*) has no effect. The inactivation of T6SS1 (by Δ*tssG1* – a baseplate complex protein) also rendered *V. proteolyticus* unable to kill *V. parahaemolyticus*. These findings demonstrated that anti-bacterial activity in *V. proteolyticus* is controlled by T6SS1 (85). In *V. coralliilyticus* MIX-effectors have been identified, and are only found in the T6SS2 (44). The MIX effector sequences indicate anti-bacterial function. The lack of MIX motifs in the T6SS1 do not preclude T6SS activity by T6SS1, as MIX containing effectors are not required for T6SS activity (83).

In summary, the T6SS in *V. coralliilyticus* is a rapid firing virulence mechanism for anti-bacterial and anti-eukaryotic attack. The diversity of potential effectors,

dynamic virulence capabilities, and genetic elements to prevent self-intoxication indicate the T6SS as an emerging, and largely unexplored, mechanism of pathogenicity in RE22Sm.

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Figure 1. Proposed mode of action of TDA. In *E. coli*, TDA acts as an electroneutral proton antiporter. At the elevated [H^+] just outside the cell membrane, the TDA carboxyl group picks up a H^+, and the neutral molecule diffuses into the cell. In the pH-neutral environment of the cytosol, TDA releases the H^+. TDA's basicity, resulting from the tropylium oxide and α-carboxyl group, allows chelation of a monovalent cation. This complex diffuses out of the cell, in aggregate resulting in an exchange of a H^+ for a monovalent cation, like K^+ (10).

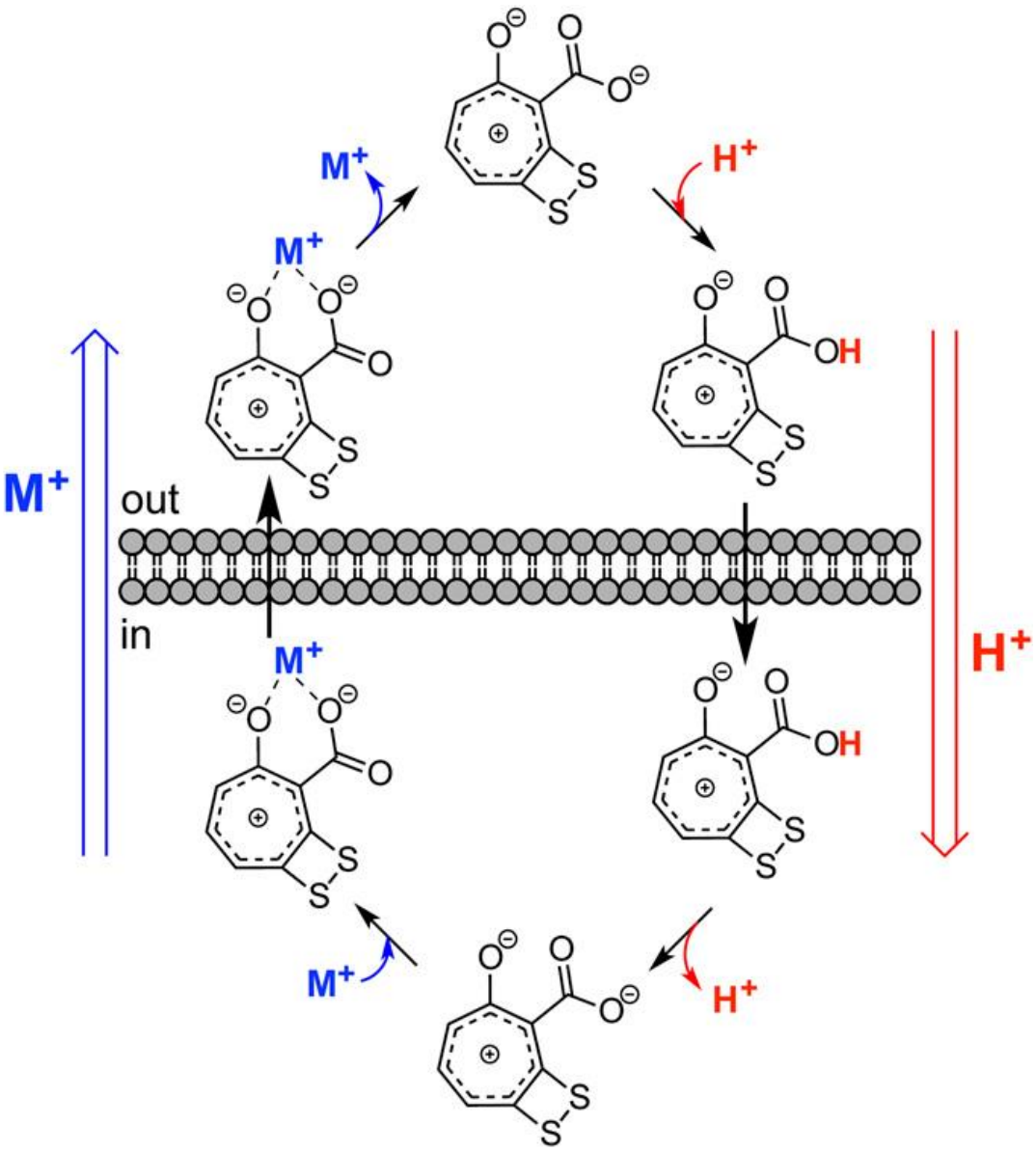
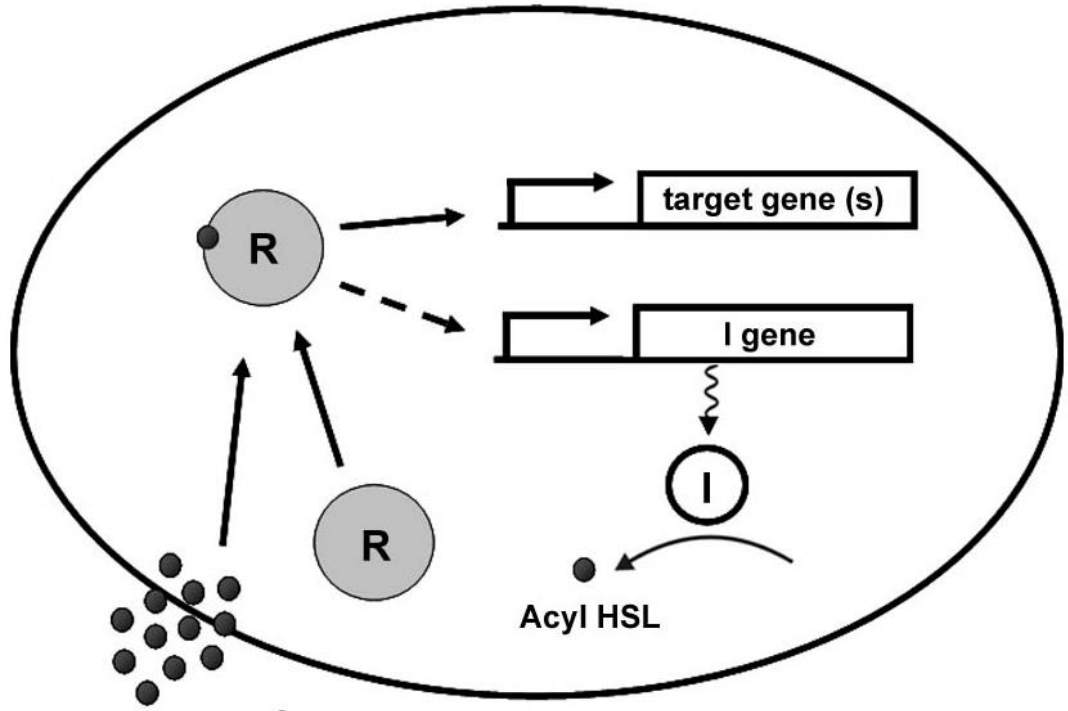
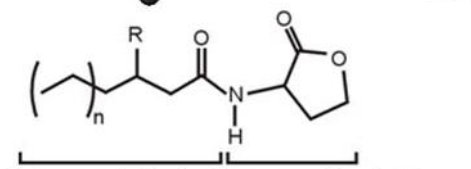

M+
M+
M+
H+
OH
M+
out
in
H+
M+
OH
M+
H+

Figure 2. Basic model of quorum sensing (QS) circuits. The ellipse represents a cell. The *I* gene represents the *luxI* homologue. R represents the acylated homoserine lactone (AHL) receptor LuxR protein. The dark unfilled circle represents the LuxI enzyme while the dark solid dots represent the AHL molecules. Stalked arrows indicate the transcription of the genes and the dotted line with arrow shows the positive feedback by the complex of the LuxR receptor and AHLs on the AHL synthase gene. The solid line with arrow depicts the function of R complex on the target genes. Squiggly line indicates translation of *I* gene and solid curved line with arrow indicates enzymatic function of *I* gene. The left corner shows the basic structure of a typical AHL molecule. *N* can vary from 4 to 18. *R* can be oxo, OH or H (21).

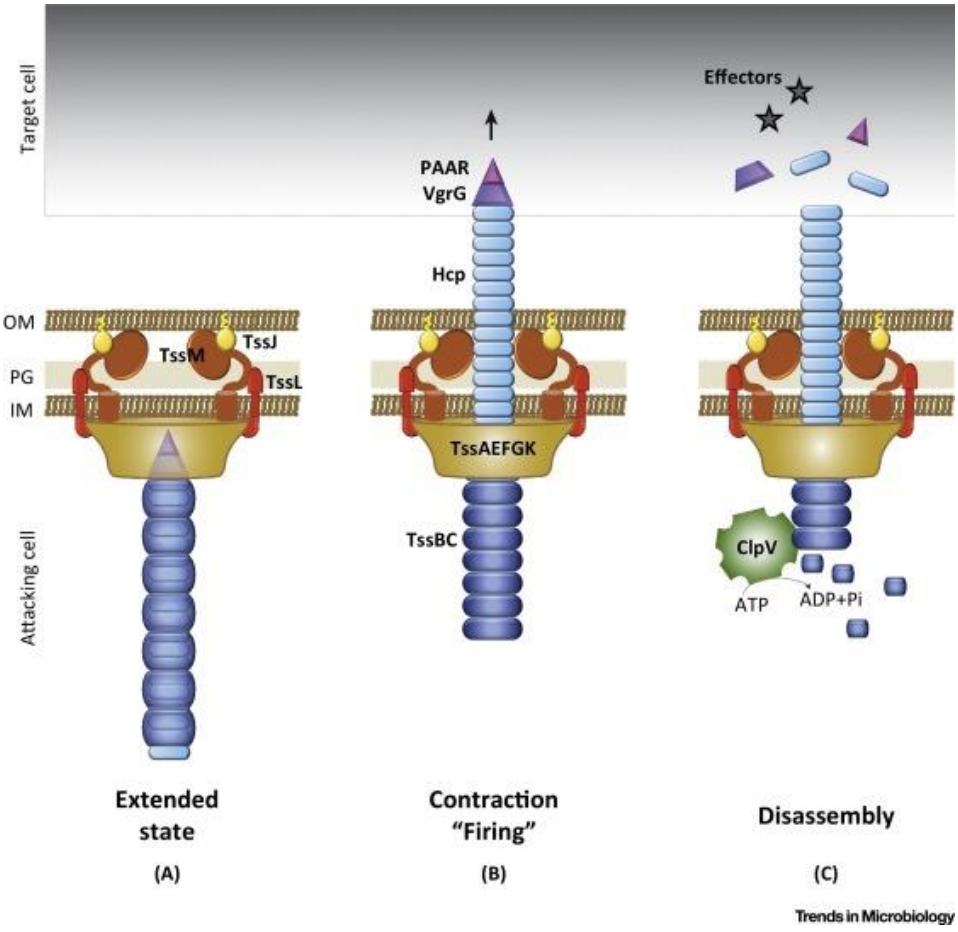




fatty acyl chain homoserine lactone

General acyl HSL

Figure 3. (A) The extended or 'primed to fire' machinery is assembled from cytoplasmic and membrane components. The membrane complex, which may initiate T6SS assembly at the inner membrane, contains TssJ, TssL, and TssM, represented in yellow, red and orange respectively. A putative baseplate-like structure, formed by TssAEFGK and represented in brown, sits at the cytoplasmic face of the inner membrane. Upon VgrG, within the baseplate, an elongated tubular structure of Hcp hexamers (light blue) is built and extends into the cytoplasm, encompassed in a TssBC sheath (blue). (B) The second step, 'firing', corresponds to sheath contraction and propels the inner tube towards the target cell. PAAR and VgrG, represented in pink and purple triangles respectively, form the puncturing device responsible for membrane perforation prior to effector delivery. (C) Once effectors (grey stars) are delivered into the target cell, the contracted sheath is disassembled by ClpV (green hexamers). Abbreviations: IM, inner membrane; OM, outer membrane; PG, peptidoglycan (67).


Target cell
Effectors
PAAR
VgrG
Hcp
OM
PG
IM
TssM
TssJ
TssL
TssAEFGK
TssBC
ClpV
ATP
ADP+Pi
Attacking cell
Extended state
(A)
Contraction "Firing"
(B)
Disassembly
(C)
Trends in Microbiology

Manuscript I

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Title: Two Type VI Secretion Systems in *Vibrio coralliilyticus* RE22Sm exhibit differential target specificity for bacteria prey and oyster larvae

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Key Words: *Vibrio coralliilyticus*, Type VI Secretion System, Hemolysin co-regulated protein, Valine glycine repeat protein G, Vibriosis, Bivalve aquaculture, Virulence.

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Abstract

Vibrio coralliilyticus is an extracellular bacterial pathogen and a causative agent of vibriosis in larval oysters. Host mortality rates can quickly reach 100% during vibriosis outbreaks in oyster hatcheries. Type VI Secretion Systems (T6SS) are rapidly polymerizing, contact dependent injection apparatus for prey cell intoxication and play important roles in pathogenesis. DNA sequencing of *V. coralliilyticus* RE22Sm indicated the likely presence of two functional T6SSs with one on each of two chromosomes. Here, we investigated the antibacterial and anti-eukaryotic roles of the two T6SSs (T6SS1 and T6SS2) against *E. coli* Sm10 cells and *Crassostrea virginica* larvae, respectively. Mutations in *hcp* and *vgrG* genes were created and characterized for their effects upon bacterial antagonism and eukaryotic host virulence. Mutations in *hcp1* and *hcp2* resulted in significantly reduced antagonism against *E. coli* Sm10, with the *hcp2* mutation demonstrating the greater impact. In contrast, mutations in *vgrG1* or *vgrG2* had little effect on *E. coli* killing. In eastern oyster larval challenge assays, T6SS1 mutations in either *hcp1* or *vgrG1* dramatically attenuated virulence against *C. virginica* larvae. Strains with restored wild type *hcp* or *vgrG* genes reestablished T6SS-mediated killing to that of wild type *V. coralliilyticus* RE22Sm. These data suggest that the T6SS1 of *V. coralliilyticus* RE22Sm principally targets eukaryotes and secondarily bacteria, while the T6SS2 primarily targets bacterial cells and secondarily eukaryotes. Attenuation of pathogenicity was observed in all T6SS mutants, demonstrating the requirement for proper assembly of the T6SS systems to maintain maximal virulence.

Importance

Vibriosis outbreaks lead to large-scale hatchery losses of oyster larvae (product and seed) where *Vibrio* sp. associated losses of 80 to 100 percent are not uncommon. Practical and proactive biocontrol measures can be taken to help mitigate larval death by *Vibrio* sp. by better understanding the underlying mechanisms of virulence in *V. coralliilyticus*. In this study, we demonstrate the presence of two Type VI Secretion Systems (T6SS) in *V. coralliilyticus* RE22Sm and interrogate the roles of each T6SS in bacterial antagonism and pathogenesis against a eukaryotic host. Specifically, we show that the loss of T6SS1 function results in the loss of virulence against oyster larvae.

Introduction

Bacterial diseases in aquatic environments negatively affect development and advancement of aquaculture systems throughout the world (1–3). *Vibrio* species are among the most common bacterial pathogens in marine aquaculture settings (4). *Vibrio coralliilyticus* RE22Sm (formerly classified as *V. tubiashii* RE22) is a Gram-negative motile marine bacterium and a member of the *Vibrionaceae* within the γ-proteobacteria class (5). *V. coralliilyticus* is a bacterial pathogen of larval eastern (*Crassostrea virginica*) and Pacific oysters (*Crassostrea gigas*) and has been associated with major disease outbreaks in hatcheries, causing shortages in seed oysters for commercial shellfish producers (1, 6). Mortality from *V. coralliilyticus* induced vibriosis can rapidly reach 100% in larval rearing tanks and contributes to significant economic losses to bivalve aquaculture worldwide (7). Historically, antimicrobial agents have been used to combat disease outbreaks in aquaculture (8). However, their usage is discouraged due to rapidly emerging antibiotic resistance and the toxicity of many of these agents (9–11). Improved knowledge on mechanisms of *Vibrio* pathogenicity in oysters would be useful in developing alternative disease management strategies.

Previous studies of virulence factors employed by *V. coralliilyticus* strains have mainly focused on extracellular enzyme function, as it was thought to be the driving force of pathogenicity (12). In addition to protease production, the annotated genome for RE22Sm (13) provides evidence for additional potential virulence factors, including a Type 3 Secretion System (T3SS), an RtxA-like toxin with its dedicated Type 1 Secretion System (T1SS), a pore-forming hemolysin (homologous to the Vah1

hemolysin of *Vibrio anguillarum*), a phospholipase hemolysin (homologous to the Plp hemolysin of *V. anguillarum*), several secreted proteases, and two Type VI Secretion Systems (T6SS) (14, 15).

The T6SS is a contact-mediated bacterial nanomachine composed of thirteen conserved proteins that inject effector proteins directly into a eukaryotic or bacterial cell (16). Many effector proteins translocated into the host/prey cell are bound as cargo to the proteins that constitute the physical T6SS puncturing device. This puncturing device is comprised of the hemolysin co-regulated protein (Hcp) and the valine glycine repeat protein G (VgrG). Specialized elongated versions of Hcp and VgrG that contain effector domains may also act as effectors (17, 18). The T6SS has been shown to be vital for virulence in many organisms, including *Vibrio cholera*e, where the T6SS was first discovered (19). Against eukaryotic prey, effector proteins can modify the host cell membrane to facilitate penetration, evade the phagosome, spread intracellularly, and cause direct cytotoxic effects (20). Moreover, the T6SS may enable Gram-negative bacteria to kill and out-compete other species of bacteria that occupy a similar niche (21). Effector proteins have been shown to cause complete lysis of other Gram-negative bacterial cells via membrane-targeting phospholipases, peptidoglycan-targeting amidases and glycoside hydrolases (22). Other antimicrobial effectors have DNase activity (23). T6SS associated effectors can be detected by conserved MIX motifs (24). Endogenous immunity proteins prevent a bacterium containing a T6SS from harming sibling cells. Some species of bacteria, such as *Pseudomonas aeruginosa* and *V. cholerae*, utilize their T6SS to translocate both antimicrobial and anti-eukaryotic effectors (24). In this report, we describe two T6SSs

used by *V. coralliilyticus* RE22Sm as antibacterial and virulence factors. These findings provide new insights into the mechanisms by which RE22Sm eliminates bacterial competition and promotes pathogenesis in oyster larvae.

Materials & Methods

Bacterial strains, plasmids and growth conditions

V. coralliilyticus RE22Sm strains (Table 3) were routinely cultured in yeast peptone broth plus 3% NaCl (YP30), yeast peptone broth plus 3% Instant Ocean© sea salt (mYP30), or Marine Minimal Medium (3M) plus 5% sucrose (35), supplemented with the appropriate antibiotic(s) in a shaking water bath (200 RPM) at 27°C. Overnight cultures of *V. coralliilyticus* RE22Sm, grown in mYP30, were harvested by centrifugation (8,000 × *g*; 10 min; 4˚C), and the pelleted cells washed twice with sterile Nine Salt Solution (NSS) (100). Washed cells were resuspended to the appropriate cell densities in experimental media. *E. coli* strains were routinely cultured in LB20 (99). Antibiotics were used at the following concentrations: streptomycin, 200 µg/ml (Sm^{200}); chloramphenicol, 5 µg/ml (Cm^{5}) for *V. coralliilyticus*, and chloramphenicol, 20 µg/ml (Cm^{20}) for *E. coli*; kanamycin, 50 µg/ml (Km^{50}) for *E. coli*, kanamycin, 80 µg/ml (Km^{80}) for *V. coralliilyticus* grown in liquid media, and kanamycin 80 µg/ml (Km^{80}) for *V. coralliilyticus* grown on solid media. Agar plates were prepared using Difco Bacto© agar at 1.6%.

V. coralliilyticus RE22Sm bioinformatic analysis

V. coralliilyticus RE22Sm draft genome (LGLS00000000) was annotated by the RAST service (http://rast.nmpdr.org/rast.cgi) with default settings (101). A list of core genes and accessory components was compiled using T6SS information from *Pantoea*

ananatis (102, 103), *Edwardsiella tarda* (104), and *Vibrio cholerae* (72, 105, 106). The MIX motif used was based on the findings of Salomon *et al.* (83). RE22Sm MIX motifs were detected by The MEME Suite – Find Individual Motif Occurrences (FIMO)(107) option, using default settings.

Allelic exchange mutagenesis

The modified pDM4 plasmid containing a kanamycin resistance (Km^R) gene, pDM5, was used to construct the allelic exchange mutants (Table 3) as described by Gibson *et al.* (108). The Km resistance gene was amplified from the TOPO2.1 vector (Invitrogen) and inserted into pDM5 via the Gibson Assembly Reaction at the AgeI restriction site. pDM5 was linearized at the SacI restriction enzyme site, using SacI-HF (New England Biolabs), within the multicloning region (MCR) for all mutation destined Gibson Assemblies. The ligation mixture was introduced into *E. coli* Sm10 (containing λpir) by electroporation with the BioRad Gene Pulser II in a 2 mm cuvette (2.5 kV; 25 µF; 200 Ω). Transformants were selected by growth on $LB20Cm_{20}$ agar plates, and successful mutagenesis was confirmed by PCR screening for a novel junction between the pDM4 plasmid and the Gibson Fragment(s) from *V. coralliilyticus*. The mobilizable suicide vector was transferred from *E. coli* Sm10 into *V. coralliilyticus* RE22Sm by conjugation as previously described (109). Transconjugants were selected by utilizing the kanamycin resistance (Km^R) gene located on the suicide plasmid. The subsequent incorporation of the target gene fragments into the suicide vector was confirmed by PCR analysis using specific primers (Table 4) to screen for the novel genetic inserts into the plasmid. The double crossover transconjugants were selected for by growth on $3MSm^{200}$ +5% sucrose agar

plates for a second crossover event. Sucrose is used as the counter selective agent because pDM5 contains the *sacB* gene, which encodes levansucrase that converts sucrose to toxic levan (110). Putative allelic exchange mutants were screened for kanamycin sensitivity. The resulting RE22Sm mutants were then screened for the desired allelic exchange double crossover using PCR amplification.

Resolution of the merodiploid state has been previously described by Milton *et al.* (110–112) and subsequently modified. After growth and passage without selection, merodiploid mutants were plated on 3M agar containing 5% sucrose and appropriate antibiotics to select for the double crossover event. The merodiploid mutants were cross-picked onto mYP30Sm^{200} and mYP30Sm^{200}Km^{80}. Successful growth in the absence of kanamycin indicated a potential allelic exchange and colonies were then screened via PCR for this double crossover event.

Bacterial killing assays

Assays for determination of T6SS-mediated killing were carried out as described by Salomon *et al.* (84). Briefly, an attacker-to-prey ratio of 4:1 (MOI of 4), based on CFU/ml, was used. A mixture of attacker and prey cells was filtered onto a 0.22 μm filter and placed on appropriate solid growth media for 4 h. The filter was then removed from the agar plate and vortexed for 1 minute in 10 ml NSS, the culture supernatant serially diluted, and plated on appropriate differential media to enumerate the attacker cells and remaining prey cells. TCBS agar was used to select for *Vibrio* spp. and MacConkey agar to select for enteric organisms.

Larval oyster experimental challenges

Assays for to determine the pathogenicity of *V. coralliilyticus* wild type and mutant strains against eastern oyster larvae were performed as previously described by Zhao *et al.* (2016) with minor modifications. Larval eastern oysters (*Crassostrea virginica*) (6 to 10 days of age, 50 – 150 µm in size) were obtained from the Blount Shellfish Hatchery at Roger William University (Bristol, RI, USA), Virginia Institute of Marine Science (Gloucester Point, VA, USA) or Niantic Bay Shellfish Farm (Niantic, CT, USA), and were allowed to acclimate for 24 h at room temperature with gentle rocking. Next, ~100 oysters were placed in each well of a 6 well plate containing 5 ml of sterilized filtered artificial seawater at 2.8% salinity. Next, the pathogen, *V. coralliilyticus* RE22Sm (wild type or mutant strain was added to the challenge wells at a final concentration of 10^5 CFU/ml and incubated for 24 h. Larval oysters were fed with commercial algal paste (20,000 cells/ml; Reed Mariculture Inc., San Jose, CA, USA) in order to promote ingestion of the probiotics. Control wells will include non-treated larvae (with and without pathogen) and larvae incubated with probiotics but not with the pathogen. Each treatment was run in triplicate and each experiment was done at least two times. Larval survival was determined 20-26 h after addition of the pathogen.

The survival rate is calculated using the formula:

Survival rate (%) = 100 x (live larvae/total number of larvae)

Statistical Analysis

Two-tailed Student's *t* tests assuming unequal variance were used for all statistical analyses for all detailed experiments. *P* values of < 0.05 were considered to be statistically significant.

Results

The RE22Sm genome contains two distinct T6SSs

Two distinct T6SS associated gene clusters were identified by utilizing a bioinformatics-guided approach to survey the annotated *V. coralliilyticus* RE22Sm genome (5, 13). Initially, genes were identified using Rapid Annotation using Subsystem Technology (RAST) (27). Twenty-two genes on chromosome 1 (Table S1) spanning 27,726 bp with a G+C ratio of 42% suggested the presence of a type VI secretion system (T6SS1). Twenty-seven genes on chromosome 2 (Table S2) spanning 25,060 bp with a G+C ratio of 43.1% were suggestive of a second system, T6SS2. The G+C content of both T6SS gene clusters was slightly lower than the entire RE22Sm genome (45.8%). Genes and motifs were identified and analyzed as previously described by Solomon *et al*. (24) to identify markers for type six effector (MIX) motifs (Table 1). Five MIX motifs were located including four within the T6SS2 gene cluster. Two MIX motifs were found in both TssA2/ImpA2 and TssI2/VgrG2. An additional MIX motif was located outside the T6SS2 gene cluster in a hypothetical protein identified as a possible oxalate:formate antiporter. The top 100 hits for this protein on BLASTx exhibited >92% amino acid sequence identity and all were in *Vibrio* species. No MIX motif-containing genes were found in the T6SS1 or elsewhere in the

RE22Sm genome.

Genes for one hemolysin co-regulated protein (*hcp1* and *hcp2*) and one valine glycine repeat protein G (*vgrG1* and *vgrG2*) were detected in each T6SS (Tables S1 and S2). The amino acid sequences of Hcp1 and Hcp2 shared 24% identity (E-value $5e^{-04}$), while the amino acid sequences of VgrG1 and VgrG2 shared 30% identity (E-value $1e^{-75}$). VgrG1 shared 86% identity with the VgrG1 protein of *V. cholerae* serotype O1, which also contains a PAAR (proline, alanine, alanine, arginine) motif (40). The *V. cholerae* VgrG1 functions as an actin cross-linking toxin in eukaryotes and a toxic effector toward bacteria (19, 41, 42). The actin cross-linking domain (ACD) of *V. cholerae* is unique to this organism and was not detected in the RE22Sm VgrG1 protein. A PAAR motif is encoded in the small *paaR* gene downstream from *vgrG1* of RE22Sm (Table S1). No PAAR motif was detected in the VgrG2 of T6SS2 (Table S2), although a possible lysozyme domain was detected. Endopeptidase and lysozyme domains were detected within a single putative extracellular protein of the M23 endopeptidase family (Accession number: **CP031473.1**) in the T6SS2 gene cluster of both *V. coralliilyticus* RE22Sm and *V. coralliilyticus* BAA-450 (YB1) (99% identity to RE22Sm M23 containing protein). This 320 amino acid protein is not associated with any annotated gene or effector, but is detectable in other *Vibrio* species (13).

We examined the antibacterial activity of the RE22Sm T6SSs by combining RE22Sm cells (attacking cells) and *E. coli* Sm10 (prey cells) on filters at a multiplicity of infection (MOI) = 4 for 4 h (Fig. 1) in a standard T6SS assay (as described in the Materials and Methods section). Incubation of RE22Sm with *E. coli* Sm10

consistently resulted in a >3 log decline the *E. coli* Sm10 cells. Knockout mutations in *hcp1*, *hcp2*, *vgrG1*, and *vgrG2* were constructed and the resulting mutant strains tested for their ability to kill *E. coli* Sm10 (Fig. 2). Knockout (KO) mutations in *hcp1* (Fig. 2a) or *hcp2* (Fig. 2b) significantly reduced killing of the target *E. coli* Sm10 cells by 2-3 orders of magnitude when compared to T = 0 h *E. coli* Sm10 CFU/ml. *E. coli* Sm10 cells declined by 1.2 log ($P < 0.05$) and 0.46 log ($P < 0.01$) when incubated with the *hcp1* and *hcp2* mutants for 4 h, respectively, as compared to a decline of 3.38 log when incubated with wild type RE22Sm cells. In contrast, mutations in either *vgrG1* (Fig. 2a) or *vgrG2* (Fig. 2b) had no significant effect on the viability of the target cells (*E. coli* declines of 3.58 log and 3.15 log, respectively). *In cis* (Fig. 2c) and *in trans* (Fig. 2d) complements of *hcp1*, *hcp2*, *vgrG1*, and *vgrG2* reversed the effects of the mutations demonstrating that knockouts of these T6SS genes affect prey killing. Further, when double KO mutants of *hcp1* and *hcp2* were tested, no significant killing of the *E. coli* target cells was detected (n.s.) (decline of 0.07 log or >85% survival). *E. coli* Sm10 cells declined by 1.2 log ($P < 0.001$) when incubated with the *hcp1/2* mutant for 4 h, compared to a decline of 3.38 log when incubated with wild type RE22Sm. Double KO mutants of *vgrG1* and *vgrG2* (Fig. 3) exhibited significantly impaired killing ability ($P < 0.005$) (decline of 1.68 log or 2.1% survival) when compared to the RE22Sm control (decline of 3.03 log). *E. coli* Sm10 cells incubated with *vgrG1/2* for 4 h declined 1.8 log ($P < 0.05$) when compared to RE22Sm wild type decline of 3.38 log.

We also examined the possibility that other potential virulence-related genes might play a role in antibacterial activity. Allelic exchange mutations in the protease

genes *vcpA* and *vcpB* and the transcriptional regulatory gene *vcpR* were constructed and the resulting strains of RE22Sm tested for their ability to kill *E. coli* target cells. The *vcpA* or the *vcpB* mutations had no effect on the killing of target cells (declines of 3.49 log and 3.08 log, respectively) as compared to the RE22Sm control (decline of 3.33 log). The *vcpR* mutant reduced the *E. coli* cell density by 2.52 log, 0.81 log less killing of *E. coli* Sm10 target cells as compared to the wild type RE22Sm cells (Table 2).

Antibacterial T6SS activity of *V. coralliilyticus* RE22Sm against *Vibrio anguillarum* strains

The T6SS assay was next used to determine the ability of RE22Sm to kill *Vibrio anguillarum* NB10Sm and M93Sm (serotypes O1 and O2, respectively) (Fig. 4). Examination of each *V. anguillarum* genome revealed that NB10Sm contains T6SS elements, while M93Sm does not. With RE22Sm as the attacking cell and NB10Sm or M93Sm as prey, both strains of *V. anguillarum* exhibited sensitivity to predation by RE22Sm (Fig. 4a). NB10Sm cell density declined by 1.81 orders of magnitude from 4.1×10^8 CFU/ml to 6.37×10^6 CFU/ml ($P < 0.005$), while M93Sm CFU/ml dropped 1.32 orders of magnitude from 4.5×10^8 CFU/ml to 2.13×10^7 CFU/ml ($P < 0.01$). These results suggest a partial, strain specific susceptibility or immunity to *V. coralliilyticus* RE22Sm T6SS effectors as compared to *E. coli* Sm10.

We also tested the ability of both *V. anguillarum* strains to kill *E. coli* Sm10 using the standard T6SS killing assay (Fig. 4b). Neither strain demonstrated virulence toward *E. coli* Sm10 comparable to that of *V. coralliilyticus* RE22Sm. Cell density of Sm10 declined by 50-60% when incubated with NB10 (n.s.), while incubation with

M93Sm resulted in a ~1 log decline in Sm10 viability ($P < 0.05$).

The effect of *V. anguillarum* strains NB10Sm and M93Sm on *V. coralliilyticus* RE22Sm viability was further examined when mixed at a ratio of 4:1 (Fig. 4c). In the presence of NB10Sm (~2×10^9 CFU/ml), RE22Sm cell density increased ~1.8-fold from 5.4×10^8 CFU/ml to 9.52×10^8 CFU/ml over 4 h ($P < 0.05$) while the NB10Sm cell density of did not significantly change. In the presence of M93Sm, the RE22Sm cell density declined 2.7-fold, from 2.84×10^8 CFU/ml to 1.05×10^8 CFU/ml, over 4 h ($P < 0.05$). Interestingly, the M93Sm density declined 5.46-fold, from 1.06×10^9 CFU/ml to 1.94×10^8 CFU/ml ($P < 0.05$).

T6SS contributes to virulence against *Crassostrea virginica* larvae

The contribution of the two T6SSs found in RE22Sm to oyster larval disease was evaluated by examining the effects of mutations in the *hcp* and *vgrG* genes. Wild type and mutant strains of RE22Sm were evaluated for their ability to kill larval oysters as described by Karim *et al.* (43). Oyster larvae infected with wild-type RE22Sm (positive infection control) exhibited 48% survival. In contrast, larvae infected with the Δ*hcp1* mutant ($P < 0.001$) or the Δ*vgrG1* mutant ($P < 0.001$) were significantly attenuated in killing compared to RE22Sm wild-type control, and were not significantly different from the no treatment control (Fig. 5). Larvae infected with the Δ*hcp2* mutant ($P < 0.005$) or the Δ*vgrG2* mutant ($P < 0.005$) showed ~74% and ~84% survival, respectively. Larvae infected with the Δ*hcp1/2* double mutant ($P < 0.005$) or Δ*vgrG1/2* double mutant ($P < 0.01$) showed ~70% and ~65% survival, respectively.

In comparison, the effects of KO mutations in the two proteases, *vcpA* and *vcpB*, and their transcriptional regulator, *vcpR*, previously identified as virulence factors in

oyster pathogenesis (44), were examined. Eastern oyster larvae infected with the Δ*vcpA* ($P < 0.005$), Δ*vcpB* ($P < 0.005$), or Δ*vcpR* ($P < 0.001$) mutants survived at ~71%, 72%, and 80%, respectively (Table S3).

Taken together, these results strongly suggest that T6SS1 is essential to RE22Sm virulence against larval oysters and that T6SS2, the VcpA and VcpB proteases, and the VcpR transcriptional regulator are important to successful pathogenesis and affect virulence, but are not essential.

Discussion

T6SSs are present in many Gram-negative bacteria and provide a means for bacterial competition and pathogenesis of eukaryotes (21). The soil pathogen *Burkholderia thailandensis* contains five distinct T6SSs that encompass a range of specificities toward different cell types (45). *V. cholerae* contains a single T6SS with dual function towards bacterial and eukaryotic target cells (46). Guillemette *et al.* (15) demonstrated that a functional T6SS in *V. coralliilyticus* OCN008 was necessary to kill strains of *V. cholerae*, adding to the repertoire of T6SSs identified in *Vibrio* species (47). A proteomic analysis of *V. coralliilyticus* YB1 supernatant detected sixteen T6SS proteins – all regulatory or structural in function (48).

In this study, we present data that *V. coralliilyticus* RE22Sm produces two functionally distinct T6SSs that act as virulence factors enabling these bacteria to attack both bacterial and eukaryotic targets. Analogous to Guillemette *et al.* (15), our RE22Sm strain can also kill the related *Vibrio* species, *V. anguillarum*. Initial detection of potential T6SS genes in RE22Sm utilized genomic findings in *V. coralliilyticus* YB1 (48) (Table S4). When the complete RE22Sm T6SS1 and T6SS2

were compared to the *Vibrionaceae* family by tBLASTx, all translated proteins were highly conserved throughout. Further, a comparative genomic approach assessing *V. coralliilyticus* virulence against *C. virginica* larvae indicates that the role of T6SS varies by bacterial strain and host/prey (49). Our results indicate that T6SS is required for pathogenicity and antibacterial activity in RE22Sm. Additionally, the multifaceted nature of the two T6SSs in *V. coralliilyticus* RE22Sm may allow their use in multiple steps during infection of oysters, such as clearing commensal bacteria, modification or killing of oyster cells and escaping the phagosome to allow intracellular spread within the host (50, 51).

Salomon *et al.* (24) proposed that some T6SS effectors in *Vibrio parahaemolyticus* could be identified by the presence of MIX motifs. We applied this idea to our inspection of the T6SSs of *V. coralliilyticus* RE22Sm. Despite the high degree of conservation of T6SSs across the *Vibrionaceae,* MIX motifs are not readily detected in RE22Sm by Find Individual Motif Occurrences (FIMO)(34). Further, we know that antibacterial activity is unaffected in the Δ*vgrG2* mutant, which contains two MIX motifs (Fig. 2b), and oyster virulence is only slightly attenuated (Fig. 5b). Consequently, the presence of MIX effectors is not required for T6SS activity, but is suggested to increase T6SS efficiency (24, 52).

Our data raised the question as to the roles of the two T6SSs in *V. coralliilyticus* RE22Sm antibacterial activity. We demonstrated that a deletion of either *hcp1* or *hcp2* results in a significant decline in the ability of these RE22Sm mutant strains to kill *E. coli* Sm10 prey cells when compared to the RE22Sm wild type. Complementation of either *hcp* gene (*cis* or *trans* complementation) restored predation activity to wild type

levels. Further, knockouts of both *hcp1* and *hcp2* resulted in a near complete loss of bacterial killing. These data show that while both Hcp proteins are necessary for fully functional T6SS-mediated antibacterial activity, the loss of Hcp2 has a significantly larger effect upon predation. In contrast, deletion of either *vgrG1* or *vgrG2* has no significant effect upon predation compared to the RE22Sm wild type (Fig. 2). However, a double mutant for both *vgrG1* and *vgrG2* shows a significant decline in bacterial killing compared to the wild type RE22Sm. These data suggest that VgrG proteins contribute to predation, but only one of the two proteins is necessary for full activity. Therefore, both T6SS1 and T6SS2 possess antibacterial activity, with the loss of a functional Hcp2 having a having a somewhat larger effect on antibacterial activity than the loss of Hcp1.

VgrG switching, as described in *Serratia marcescens,* may account for the retention of function despite loss of either VgrG1 or VgrG2 is (53). Such switching capacity would allow the loaded VgrG, acting as an effector, to display preferential target specificity and the puncturing apparatus to be loaded according to the target organism (23). A second possibility is that only one complete T6SS system is necessary for antibacterial activity; however, the loss of either Hcp1 or Hcp2 has a much larger effect than the loss of either VgrG1 or VgrG2.

Guillemette *et al*. (15) examined the question of whether deletion mutations of protease genes *vtpA*, *vtpB* (renamed *vcpA* and *vcpB*) or their transcriptional regulator *vtpR* (*vcpR*) provide protection against predation by *V. cholerae* or affected T6SS-mediated killing of *V. cholerae* by *V. coralliilyticus* OCN008. They found that knockouts of *vcpA* and/or *vcpB* had no effect upon survival against killing by *V.*

cholerae or ability to kill *V. cholerae*. However, the *vcpR* mutant had reduced ability to survive attack by *V. cholerae* and lost the ability to kill *V. cholerae*. We also found that deletion of either *vcpA* or *vcpB* in *V. coralliilyticus* RE22Sm had no effect on T6SS-mediated killing of prey cells. However, deletion of *vcpR* produced modest effect on predation of *E. coli* Sm10. The KO mutation of *vcpR* reduced antibacterial activity with target cell decline of 2.52 log compared to wild type RE22Sm causing *E. coli* Sm10 cell density to decline by 3.33 log, a reduction of ~0.8 log. While we do not know the reason for the difference between effects of the *vcpR* mutation on predation, we suggest that *E. coli* Sm10 is a more vulnerable prey target than *V. cholerae*, perhaps because *V. cholerae* contains a T6SS with immunity genes (54) and *E. coli* Sm10 does not (55).

Our data also indicate that *V. coralliilyticus* RE22Sm is able to kill *V. anguillarum* strains NB10Sm and M93Sm (serotypes O1 and O2, respectively). However, both strains are significantly less sensitive to T6SS than *E. coli* Sm10. The decreased sensitivity of *V. anguillarum* to *V. coralliilyticus* T6SS-mediated predation may be due to the presence of immunity genes in their T6SS gene clusters. Tang *et al.* (2016) showed that *V. anguillarum* strains possess T6SS and are able to kill *E. coli* and *Edwardsiella tarda* (56). Our data demonstrate that NB10Sm is unable to kill either *E. coli* Sm10 (Fig. 4b) or *V. coralliilyticus* RE22Sm (Fig. 4c) in the T6SS assay, despite containing T6SS elements. However, while a search of the *V. anguillarum* M93Sm genome failed to reveal any T6SS genes, this O2 serotype strain is able to kill *E. coli* Sm10 in our T6SS assay. These results are of interest due to our initial hypothesis indicating that NB10Sm would be more virulent against *E. coli* Sm10 and RE22Sm

than M93Sm due to the presence of T6SS genes in NB10Sm. We suggest that the T6SS of NB10Sm is inactive and that M93Sm has an unknown mechanism of antibacterial activity.

Our data begin to address the major role of the two T6SSs in *V. coralliilyticus* RE22Sm virulence against oysters. The hardened tip motif, PAAR, is present in the *paaR* protein found in T6SS1, (41) allowing a wider range of potential targets, including coral, the namesake target of *V. coralliilyticus*, and possibly other eukaryotes. This idea is supported by our observation that mutants lacking either *hcp1* or *vgrG1* are completely avirulent against oyster larvae, indicating that T6SS1 is required for pathogenesis against oyster larvae. In contrast, knockouts of either *hcp2* or *vgrG2* exhibited only partially attenuated virulence, suggesting that the T6SS2 plays a more limited role in pathogenesis of oyster larvae. A similar effect on virulence has been previously reported in *P. aeruginosa*, a microbe with multiple T6SSs under the transcriptional control of RpoN (σ^{54}) (57). Further, contrary to expectations, RE22Sm mutants containing knockouts of both *hcp1* and *hcp2* or *vgrG1* and *vgrG2* were able to kill oyster larvae at greater rates than any of the single mutants in these genes. Understanding this observation will require further investigation, but does raise the possibility that other virulence genes are up-regulated when both T6SSs are knocked out.

The activities of the RE22Sm T6SSs together with other previously described virulence factors help to decode the pathogenic potential of this organism and demonstrate how this fast growing, motile organism can cause substantial mortality in an aquaculture setting. Increased understanding of *V. coralliilyticus* virulence genes

involved in oyster infection should help inform efforts to prevent larval and juvenile vibriosis.

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CS & DRN designed the study. CS created the mutant strains used in this study. CS performed all experiments under the supervision of DRN. CS and DRN wrote the manuscript with contributions and edits from DCR and MGC. Manuscript formatting was performed by CS and DRN. All authors read and approved the final version of this manuscript.

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Table 1. T6SS MIX motif search in *V. coralliilyticus* RE22Sm (40)

T6SS [a]	**Strand**	**Gene Name**	**Protein**	**Inclusive Nucleotides (GenBank: CP031473.1)**	**MIX Motif** [b]
T6SS2	+	*tssA impA2*/*vasJ2*	ImpA2	81898 – 83235	ARMGAFEWL
T6SS2	-	*tssA impA2.1*/*vasJ2.1*	ImpA2.1	71118 – 72683	GRDGAVEWL
T6SS2	-	*tssI vgrG2*	VgrG2	63092 – 65080	AEHGMWYYF
T6SS2	-	*tssI vgrG2*	VgrG2	63092 – 65080	PTWGAVYLP
T6SS2	+	No annotation	MFS_OFA_like Superfamily	41958 – 43190	LCLGILYAW

[a] No MIX motif hits for T6SS1

[b] MIX MOTIF: hRXGhhYhh; h: GAVLIPFMW (83)

Table 2. Effects of *V. coralliilyticus* protease mutants on T6SS-mediated antibacterial activity.

Strain Mixtures	**T0 CFU/mL (±1 SD)**	**T4 CFU/mL (±1 SD)**
Mixture 1		
V. coralliilyticus RE22Sm [a]	1.83E+09 (±1.6E+08)	1.03E+09 (±7.6E+08)
E. coli Sm10 [b]	5.17E+08 (±5E+07)	2.41E+05 (±6.8E+04)
Mixture 2		
V. coralliilyticus Δ*vcpA* [a]	1.43E+09 (±4.5E+08)	3.10E+09 (±1.5E+08)
E. coli Sm10 [b]	5.33E+08 (±1.82E+07)	1.73E+05 (±4.39E+04)
Mixture 3		
V. coralliilyticus Δ*vcpB* [a]	1.30E+09 (±4.6E+08)	5.33E+09 (±4.8E+08)
E. coli Sm10 [b]	6.00E+08 (±3.6E+07)	5.00E+05 (±3.5E+04)
Mixture 4		
V. coralliilyticus Δ*vcpR* [a]	1.67E+09 (±8.9E+08)	6.20E+08 (±1.37E+08)
E. coli Sm10 [b]	4.44E+08 (±3.17E+08)	1.34E+06 (±5.87E+05)

[a] Predator cells were mixed in a ratio of 4:1 with [b] prey cells as described in the Materials and Methods section. The data are the average of 3 biological replicates (experiments); each experiment had three technical replicates.

Table 3. Bacterial strains and plasmids used in this study

Strain	Description	Resistance	Reference
V. coralliilyticus			
RE22	Wild-type isolate from oyster larvae		Estes *et al.* 2004
RE22Sm	Spontaneous Sm^r mutant of RE22	Sm^r	Zhao *et al.* 2016
RE22Sm-GFP	Sm^r Cm^r; RE22Sm (pRhokHi-2-*gfp*)	Sm^r Cm^r	Zhao *et al.* 2016
RE22SmKm	Sm^r Km^r mutant of RE22 harboring an empty pSUP203 shuttle vector	Sm^r Km^r	This study
RE22Δ*hcp1*	Sm^r Km^r; allelic exchange mutation of *hcp1* using pDM5; $T6SS^{-/-}$	Sm^r Km^r	This study
RE22Δ*hcp2*	Sm^r Km^r; allelic exchange mutation of *hcp2* using pDM5; $T6SS^{-/-}$	Sm^r Km^r	This study
RE22Δ*vgrG*1	Sm^r Km^r; allelic exchange mutation of *vgrG*1 using pDM5; $T6SS^{-/-}$	Sm^r Km^r	This study
RE22Δ*vgrG*2	Sm^r Km^r; allelic exchange mutation of *vgrG2* using pDM5; $T6SS^{-/-}$	Sm^r Km^r	This study
RE22 Δ*hcp1*Δ*hcp2*	Sm^r Cm^r; Allelic exchange mutation of *hcp1* and *hcp2* using pDM5; $T6SS^{-/-}$	Sm^r Cm^r Km^r	This study
RE22 Δ*vgrG1*Δ*vgrG2*	Sm^r Cm^r; Allelic exchange mutation of *vgrG1* and *vgrG2* using pDM5; $T6SS^{-/-}$	Sm^r Cm^r Km^r	This study
RE22 Δ*vcpA*	Sm^r Km^r; allelic exchange mutation of *vcpA* using pDM4	Sm^r Cm^r	This study

RE22 Δ*vcpB*	Sm^r Km^r; allelic exchange mutation of *vcpB* using pDM4	Sm^r Cm^r	This study
RE22 Δ*vcpR*	Sm^r Km^r; allelic exchange mutation of *vcpR* using pDM4	Sm^r Cm^r	This study
V. anguillarum			
NB10SmKm	Spontaneous Sm^r mutant of strain NB10 harboring an empty pSUP203 shuttle vector	Sm^r Km^r	This study
M93SmKm	Spontaneous Sm^r mutant of strain M93 harboring an empty pSUP203 shuttle vector (contains Km resistance gene)	Sm^r Km^r	This study
E. coli			
Sm10	*Thi thr leu tonA lacY supE recA* RP4-2 Tc::Mu::Km (λ)	Km^r	Simon *et al*., 1983
Sm100	Sm10 harboring pDM5 plasmid	Km^r Cm^r	This study
S122	Sm10 harboring pSUP202P-*gfp*(ORF)	Km^r	Zhao *et al*. 2016
CS01	Sm10 harboring pDM5-*hcp1*		
CS02	Sm10 harboring pDM5-*hcp2*	Km^r Cm^r	This study
CS03	Sm10 harboring pDM5-*vgrG1*	Km^r Cm^r	This study
CS04	Sm10 harboring pDM5-vgrG2	Km^r Cm^r	This study
CS05	Sm10 harboring pDM4-*vcpA*	Cm^r	This study
CS06	Sm10 harboring pDM4-*vcpB*	Cm^r	This study
CS07	Sm10 harboring pDM4-*vcpR*	Cm^r	This study

Plasmids			
pDM4	Cm^r; suicide vector with R6K origin and *sacB*	Cm^r	Milton, 1996
pDM5	Cm^r Km^r; suicide vector with R6K origin and *sacB*	Cm^r Km^r	This study
pSUP202P	Ap^r Cm^r Tc^r; broad host shuttle vector	Ap^r Cm^r Tc^r	Simon *et al*., 1983
pSUP203	Ap^r Cm^r Tc^r Km^r; broad host shuttle vector	Ap^r Cm^r Tc^r Km^r	This study
pRhokHi-2-*gfp*	pRhokHi-2-FbFP with *gfp* under the control of P*aphII*	Cm^r	Zhao *et al*. 2016

Table 4. Primers used in this study

Primer	**Sequence (5' to 3', underlined sequences are engineered for Gibson Assembly sites in pDM5)**	**Description**
PmH37	tgtggaatcccgggagagctCAATGTGAACAGACTATTCAAAC	For *hcp1* insertional mutation, 5' forward
PmH38	tgtgcaacacCGTAAAGGCACAGCAGAC	For *hcp1* insertional mutation, 5' reverse
PmH39	tgcctttacgGTGTTGCACACATTGAAG	For *hcp1* insertional mutation, 3' forward
PmH40	gcatgcgggtaacctgagctCAGATCGTCTTCAACATTG	For *hcp1* insertional mutation, 3' reverse
Pmh41	tgtggaatcccgggagagctCAGCAGTCGAAGTAACTTTC	For *hcp2* insertional mutation, 5' forward
Pmh42	aacgctgaccCGAATCTTTCTCTCTAACC	For *hcp*

		insertional mutation, 5’ reverse
Pmh43	gaaagattcgGGTCAGCGTTGTTTGCGTTAC	For *hcp2* insertional mutation, 3’ forward
Pmh44	gcatgcgggtaacctgagctGAAGGAGATCAACATGGCTTC	For *hcp2* insertional mutation, 3’ reverse
PmG45	tgtggaatcccgggagagctCCTTTCAGTTCGCCTAGTAC	For *vgrG1* insertional mutation, 5’ forward
PmG46	tgataaagtcGTTTATTCCACGAGTAGGTC	For *vgrG1* insertional mutation, 5’ reverse
PmG47	tggaataaacGACTTTATCAGTGGGAGG	For *vgrG1* insertional mutation, 3’ forward
PmG48	gcatgcgggtaacctgagctGTAACAGACTCATTATTTCAAG	For *vgrG1* insertional

		mutation, 3’ reverse
Pmg49	tgtggaatcccgggagagctGCTACGTTGCCACCTTTAATC	For *vgrG2* insertional mutation, 5’ forward
Pmg50	tctagtgatgCTCCTTACTCATTGCCTG	For *vgrG2* insertional mutation, 5’ reverse
Pmg51	gagtaaggagCATCACTAGACATTTCCTGCG	For *vgrG2* insertional mutation, 3’ forward
Pmg52	gcatgcgggtaacctgagctCAGGGTAGTGGCCATGAATTTC	For *vgrG2* insertional mutation, 3’ reverse
PmA53	tgtggaatcccgggagagctTACCAGTTACAGCCGCAG	For *vcpA* insertional mutation, 5’ forward
PmA54	acctgaagtaCAACAAAAAAGTCTACCATGTAAAC	For *vcpA* insertional mutation, 5’

		reverse
PmA55	tttttttgttgTACTTCAGGTAGCACAGC	For *vcpA* insertional mutation, 3' forward
PmA56	gcatgcgggtaacctgagctTAGAAGGCACGGTTGTAC	For *vcpA* insertional mutation, 3' reverse
PmB57	tgtggaatcccgggagagctTTCTGAGTAACCGAATACGTTGAC	For *vcpB* insertional mutation, 5' forward
PmB58	tgactgtggaGCACGAAGTCAGCCATGG	For *vcpB* insertional mutation, 5' reverse
PmB59	gacttcgtgcTCCACAGTCACAACGTTATC	For *vcpB* insertional mutation, 3' forward
PmB60	gcatgcgggtaacctgagctTCTGGGCTGAATTCTCAG	For *vcpB* insertional mutation, 3' reverse

PmR11	tgtggaatcccgggagagctTATACAACTCAATTGGCAAGG	For *vcpR* insertional mutation, 5' forward
PmR12	gattgctttgATGCGATTTCCATCAGTTG	For *vcpR* insertional mutation, 5' reverse
PmR13	gaaatcgcatCAAAGCAATCGAGCGTGG	For *vcpR* insertional mutation, 3' forward
PmR14	gcatgcgggtaacctgagctTCTAGGTAGCTTTGTGTCAG	For *vcpR* insertional mutation, 3' reverse
Chcp1 FP	ttatgtctattgctggtttaACAGAGCATTATTGGCGTG	For *hcp1 in trans* complement in pSUP202, forward
Chcp1 RP	tgcttccggtagtcaataaaCTAGGATAGTTTTCAGATCGTC	For *hcp1 in trans* complement in pSUP202,

		reverse
Chcp2 FP	ttatgtctattgctggtttaTTAAGCAGCAGTCGAAGTAACTTTC	For *hcp2 in trans* complement in pSUP202, forward
Chcp2 RP	tgcttccggtagtcaataaaTCCTGTCGCCCAAAACCAAG	For *hcp2 in trans* complement in pSUP202, reverse
CvgrG1 FP	ttatgtctattgctggtttaAGTTTACCTTCACAATGG	For *vgrG1 in trans* complement in pSUP202, forward
CvgrG1 RP	tgcttccggtagtcaataaaGACAATAACCTAGATTACCTAC	For *vgrG1 in trans* complement in pSUP202, reverse
CvgrG2 FP	GTCGCTGCCTTCAACTGTGATGGAAG	For *vgrG2 in trans* complement in pSUP202,

		forward
CvgrG2 RP	GGCCACTACCCTGAACTTCAAATTTAA	For *vgrG2 in trans* complement in pSUP202, reverse

Figure 1. Determination of the *V. coralliilyticus* RE22Sm (attacking cell) T6SS-mediated antibacterial activity against *E. coli* Sm10 (prey cell) when incubated on a filter for 4 h at 27°C with a 4:1 predator: prey ratio (MOI = 4). Starting RE22 cell density was ~2×10^9 CFU/ml and starting *E. coli* Sm10 cell density was ~5×10^8 CFU/ml. The data are the average of 3 biological replicates (experiments); each experiment had three technical replicates. Error bars represent ±1 standard deviation (SD). Statistical analysis by Student's T-test. ns = not significant, **** = $P < 0.001$

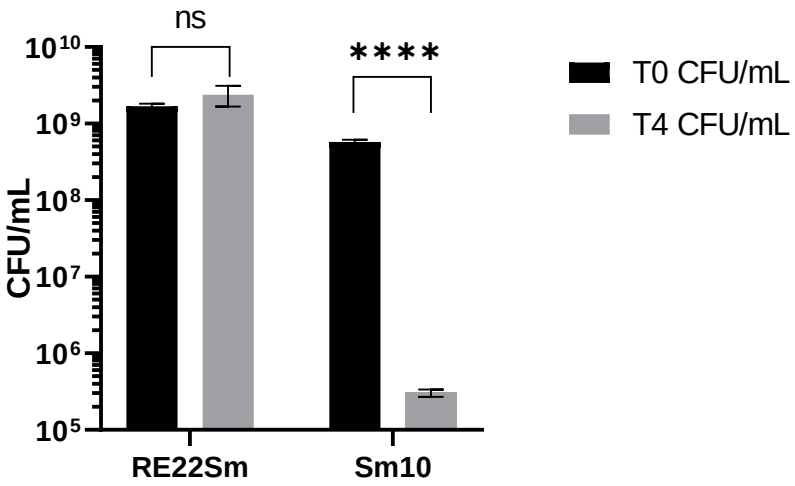

ns
****
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
CFU/mL
RE22Sm
Sm10
T0 CFU/mL
T4 CFU/mL

Figure 2. T6SS-mediated anti-bacterial activity of *V. coralliilyticus* RE22Sm wild type and T6SS mutant strains against *E. coli* Sm10 prey cells. Each group of two bars shows the cell density (CFU/ml) of the *E. coli* Sm10 prey cells at T= 0 h (black bar) and T= 4 h (grey bar) after being mixed with attacking *V. coralliilyticus* wild type (RE22Sm) or T6SS mutant strains. (A) T6SS killing activity of RE22Sm mutant strains Δ*hcp1*, Δ*vgrG1*, and RE22Sm wild-type control. (B) T6SS2 killing assay by RE22Sm mutant strains Δ*hcp2*, Δ*vgrG2*, and RE22Sm wild-type control. (C) T6SS killing assay by RE22Sm T6SS mutant revertant, strains and RE22Sm wild-type control. (D) T6SS killing assay by RE22Sm T6SS mutant *in-trans* complement strains, and RE22Sm wild-type control. All data are averages of at least 3 experiments; error bars show ±1 SD; ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$ (Statistical analysis by unpaired Student's T-test).

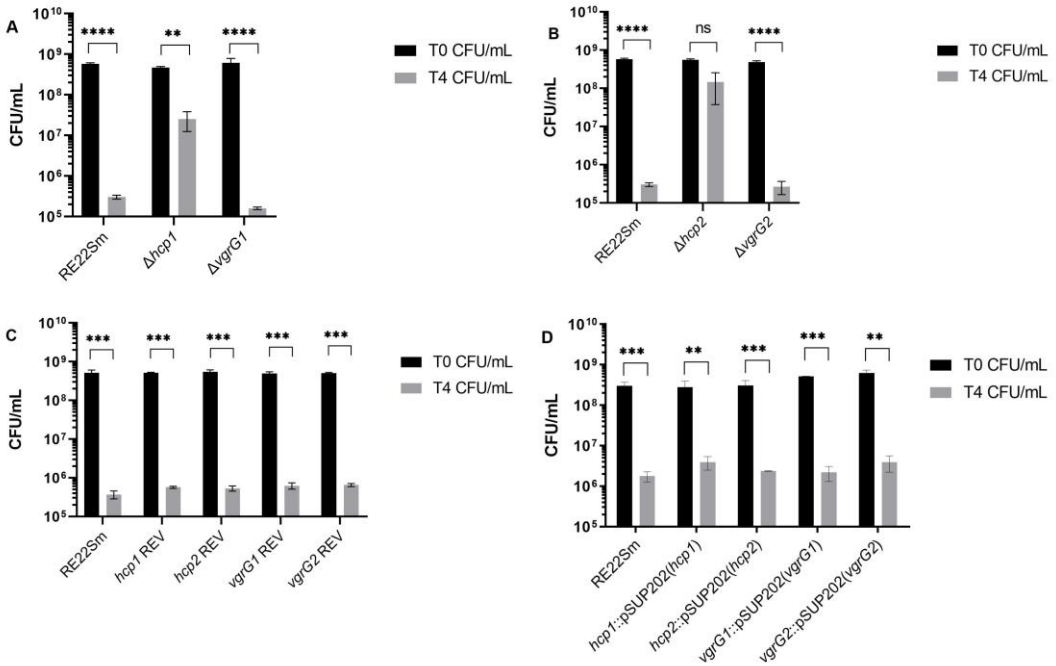

A
B
C
D
CFU/mL
T0 CFU/mL
T4 CFU/mL
RE22Sm
Δhcp1
ΔvgrG1
Δhcp2
ΔvgrG2
hcp1 REV
hcp2 REV
vgrG1 REV
vgrG2 REV
hcp1::pSUP202(hcp1)
hcp2::pSUP202(hcp2)
vgrG1::pSUP202(vgrG1)
vgrG2::pSUP202(vgrG2)
ns

Figure 3. T6SS killing activity of *V. coralliilyticus* RE22Sm wild-type and T6SS double mutants against *E. coli* Sm10 prey cells. Each group of two bars shows the cell density (CFU/ml) of the *E. coli* Sm10 prey cells at T= 0 h (black bar) and T =4h (grey bar) after being mixed with attacking *V. coralliilyticus* wild type (RE22Sm) or T6SS mutant strains. Average of 3 experiments; error bars indicate ±1 SD; ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$ (Statistical analysis by unpaired Student's T-test).

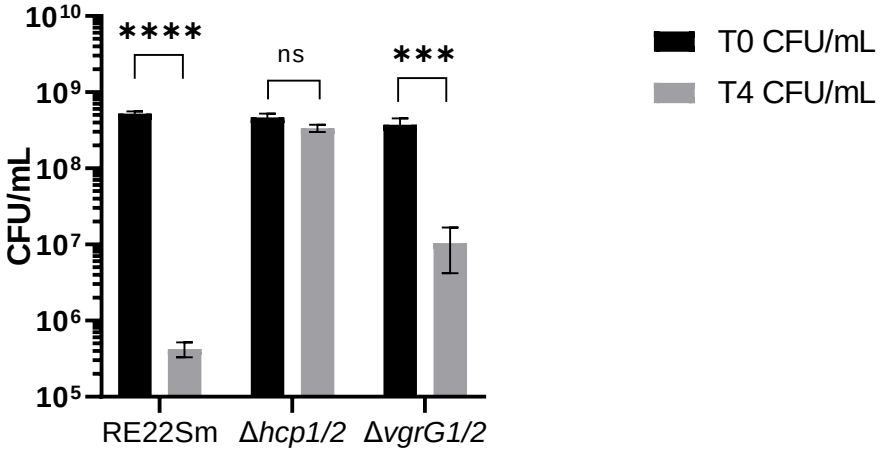

10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
CFU/mL
****
ns
***
RE22Sm
Δ*hcp1/2*
Δ*vgrG1/2*
T0 CFU/mL
T4 CFU/mL

Figure 4. T6SS killing activity of *V. coralliilyticus* RE22Sm, *V. anguillarum* NB10Sm, and *V. anguillarum* M93Sm against *E. coli* Sm10 and *Vibrio* prey cells. Each group of four bars indicate attacker (first two bars) and prey cell (second two bars) cell density at T= 0 h (black bars) and 4 h (grey bars) (A) The ability of RE22Sm to kill serotype O1 (NB10Sm) and O2 (M93Sm) strains of *V. anguillarum*. (B) The ability of *V. anguillarum* NB10Sm and M93Sm to kill *E. coli* Sm10. (C) The ability of *V. anguillarum* NB10Sm and M93Sm to attack *V. coralliilyticus* RE22Sm. The data are the averages of at least 3 experiments; the error bars indicate ±1 SD; ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$ (Statistical analysis by unpaired Student's T-test).

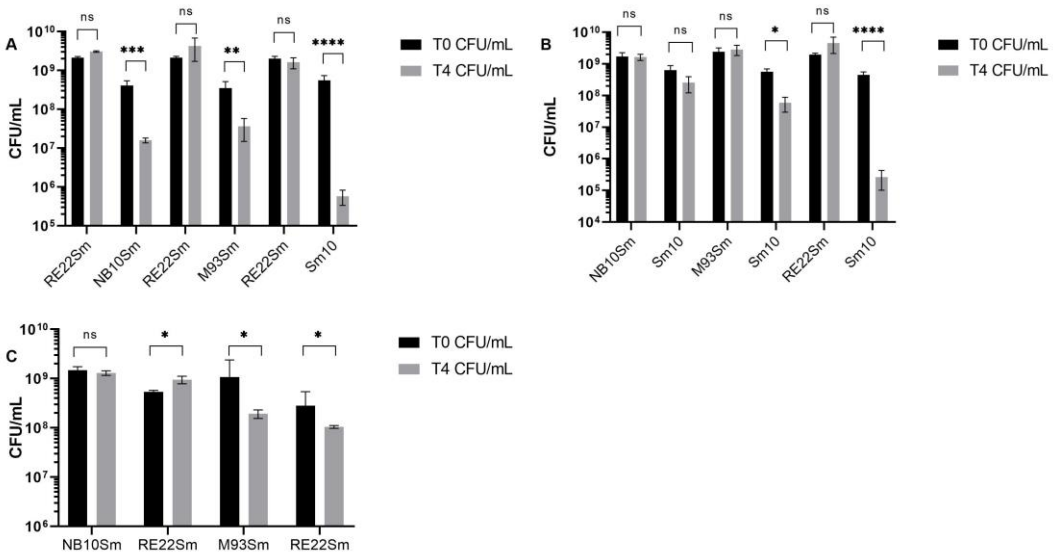
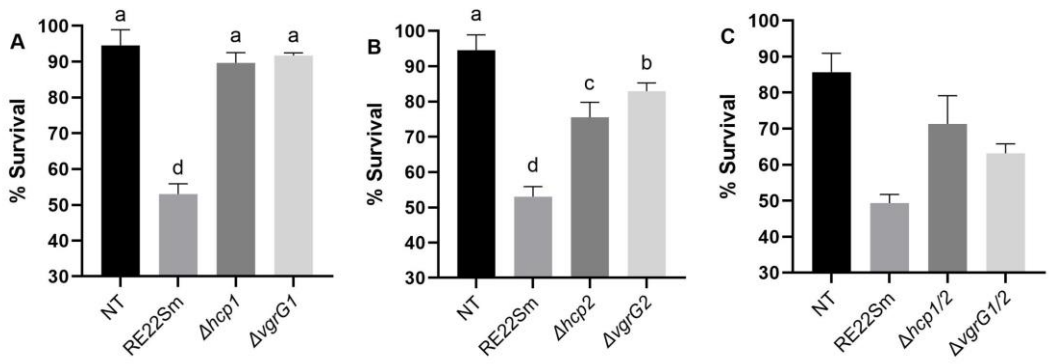

A
B
C
CFU/mL
T0 CFU/mL
T4 CFU/mL
ns
*
**
***
****
RE22Sm
NB10Sm
M93Sm
Sm10

Figure 5. Oyster larvae survival after challenge *with V. coralliilyticus* RE22Sm. Oyster larvae were exposed to RE22Sm wild type and mutant strains (1×10^5 CFU/ml) for 24 h. Oyster larvae treated with artificial seawater served as the negative control. Larval survival (% ±1 SD) was determined after 24 h challenge. (A) *V. coralliilyticus* RE22Sm wild type and T6SS1 mutants (Δ*vgrG1* and Δ*hcp1*) tested for virulence against larval oysters. (B) *V. coralliilyticus* RE22Sm wild type and T6SS2 mutants (Δ*vgrG2* and Δ*hcp2*) tested for virulence against larval oysters. (C) *V. coralliilyticus* RE22Sm wild type and T6SS double mutants *Δhcp1/2* and *ΔvgrG1/2* tested for virulence against larval oysters. Average of at least 3 biological replicates; the error bars indicate ±1 SD; different letters indicate statistical between among groups, a = $P > 0.05$, b = $P < 0.05$, c = $P < 0.01$, d = $P < 0.005$ (Statistical analysis by unpaired Student's T-test, $P < 0.05$).


A
% Survival
100
90
80
70
60
50
40
30
a
d
a
a
NT
RE22Sm
Δhcp1
ΔvgrG1
B
% Survival
a
d
c
b
NT
RE22Sm
Δhcp2
ΔvgrG2
C
% Survival
NT
RE22Sm
Δhcp1/2
ΔvgrG1/2

Supplemental Data

Table S1. T6SS1 genes identified on chromosome 1 (GenBank: CP031472.1)

Inclusive base pairs		Strand	Gene	Homologue	Annotation	Putative Function
675003	678314	-	*tssM*	*vasK, icmF*	IcmF-related protein	Anchoring T6SS to cell wall
678389	679591	-		*impK, vasF*	Outer membrane protein ImpK/VasF, OmpA/MotB domain	Unknown function
679600	680928	-	*tssK*	*impJ, vasE*	VasE Superfamily	Unknown function
680952	681431	-	*tssJ*	*vasD, lip*	Type VI secretion lipoprotein/VasD	Anchoring T6SS to cell wall
681434	682144	-	*tagH*	*impI*	Uncharacterized protein ImpI/VasC	FHA domain-containing protein, post-translational regulation, putative nuclear signaling domain
682189	682338	-			Hypothetical protein	Unknown
682382	683101	-			FIG01199604: hypothetical protein	ABC transporter substrate-binding protein [*Vibrio coralliilyticus*]/ SBP bac3 superfamily
683092	684090	-			ABC-type uncharacterized transport system, periplasmic component	ABC transporter substrate-binding protein [*Vibrio coralliilyticus*]
684090	686621	-		*mdtG*	putative inner membrane transport protein	Major Facilitator Superfamily; drug efflux system protein
686659	689268	-	*tssH*	*clpV, vasG*	ClpB protein	ATPase/effector chaperon/recycling TssB/TssC
689346	690272	-	*tssG*	*impH, vasB*	Uncharacterized protein ImpH/VasB	Unknown function/ intracellular trafficking, secretion and vesicular transport
690296	692044	-	*tssF*	*impG, vasA*	Protein ImpG/VasA - phage tail protein needed for Hcp assembly	Unknown function - necessary for Hcp assembly
692040	692459	-	*tssE*	*impF, vasS*	FIG01286925:	Essential baseplate

					hypothetical protein	protein similar to T4 phage gp25 proteins
692462	693853	-	*tssC*	*impC, vipB*	Uncharacterized protein ImpD	Homologous to T4 phage contractile tail sheath proteins
693918	695393	-	*tssC*	*impC, vipB*	Uncharacterized protein ImpC	Homologous to T4 phage contractile tail sheath proteins
695393	695896	-	*tssB*	*impB, vipA*	Uncharacterized protein ImpB	Homologous to T4 phage contractile tail sheath proteins
695919	696437	-	*tssD*	*hcp1*	Uncharacterized protein ImpD	Effector/Structure: Homologous to T4 phage tube
696475	697875	-	*tssA*	*impA, vasJ*	Uncharacterized protein ImpA	Unknown function - *impA* N terminal domain
698275	698421	-	*paaR*		PAAR containing protein	Protein with a PAAR motif associated with VgrG piercing structure
698574	699140	-			Twin-arginine translocation pathway signal	Hypothetical protein
699148	701127	-	*tssI*	*vgrG1*	VgrG protein	Effector/structure: forms the T6SS piercing structure
701325	702128	-			Hypothetical protein	Hypothetical protein

Table S2. T6SS2 genes identified on chromosome 2 (GenBank: CP031473.1)

Inclusive base pairs		Strand	Gene	Homologue	Annotation	Putative Function
87561	88349	-		*dotU*	Outer membrane protein ImpK/VasF, OmpA/MotB domain	IcmF associated DotU inner membrane anchoring protein
86281	87561	-	*tssK*	*impJ, vasE*	Uncharacterized protein ImpJ/VasE	Unknown function
85778	86224	-	*tssJ*	*vasD, lip*	Type VI secretion lipoprotein/VasD	Anchoring T6SS to cell wall
84349	85731	-	*tagH*	*impI*	Uncharacterized protein ImpI/VasC	FHA domain-containing protein, post-translational regulation
83734	84333	-			FIG01199688: hypothetical protein	Outer membrane protein with beta-barrel domain
81901	83235	+	*tssA*	*impA, vasJ*	Uncharacterized protein ImpA	Unknown function - *impA* N terminal domain & DUF1043 superfamily
78503	81895	+	*tssM*	*vasK, icmF*	IcmF-related protein	Anchoring T6SS to cell wall
78013	78459	+			Transcriptional regulator, AsnC family	
76943	77938	-	*tssG*	*impH, vasB*	Uncharacterized protein ImpH/VasB	Unknown function
75198	76943	-	*tssF*	*impG, vasA*	Protein ImpG/VasA	Unknown function
74769	75176	-	*tssE*	*impF, vasS*	Uncharacterized protein similar to VCA0109	Essential baseplate protein similar to T4 phage gp25 proteins
73278	74702	-	*tssC*	*impC, vipB*	Uncharacterized protein ImpC	Homologous to T4 phage contractile tail sheath proteins
72711	73217	-	*tssB*	*impB, vipA*	Uncharacterized protein ImpB	Homologous to T4 phage contractile tail sheath proteins
71118	72680	-	*tssA*	*impA, vasJ*	Uncharacterized protein ImpA	Unknown function
69102	71111	-	*tagE*	*pknA, ppkA*	Serine/threonine protein kinase	Serine/threonine kinase, post-translational regulation

67955	68905	-			FIG01200163: hypothetical protein	No conserved domains
66975	67955	-		*tagAB*	Pentapeptide repeat family protein	Domain of unknown function
65675	66991	-			FIG01200268: hypothetical protein	No conserved domains
65170	65652	-			FIG01199591: hypothetical protein	No conserved domains
63092	65167	-	*tssI*	*vgrG2*	VgrG protein	Effector/structure: forms the T6SS piercing structure (potential lysozyme domain)
62502	63017	-	*tssD*	*hcp2*	Hcp protein	Effector/Structure: Homologous to T4 phage tube
59370	62039	+	*tssH*	*clpV*	ClpV protein	MULTISPECIES: ClpV family T6SS ATPase [*Vibrio*]
57746	59380	+			Sigma-54 dependent transcriptional regulator	
52921	57684	-		*tagL*	Hypothetical protein	OmpA family protein [*Vibrio coralliilyticus*]
51663	52925	-			Hypothetical protein	No conserved domains
49984	51636	-			FIG01201986: hypothetical protein	No conserved domains
48162	49121	+		*L376_02862*	Cell wall endopeptidase, family M23/M37	Protein with a peptidase M23 domain, putative endopeptidase effector

Table S3. Larval oyster survival after challenge with wild type and mutant strains of *V. coralliilyticus* RE22Sm

Treatment [a]	**% Mean Survival (±1 SD)** [b]
No Treatment	91.80% ± 4.36
RE22Sm	48.60% ± 2.89
RE22Sm Δ*vcpA*	70.58% ± 0.86
RE22Sm Δ*vcpB*	72.09% ± 5.27
RE22Sm Δ*vcpR*	80.15% ± 1.24

[a] Oyster larvae were exposed to RE22Sm wild type and mutant strains (1×10^5 CFU/ml) for 24 h. Oyster larvae treated with artificial seawater served as the negative control.

[b] Larval survival (% ±1 SD) was determined after 24 h challenge. The survival rate is calculated using the formula:

Survival rate (%) = 100 x (live larvae/total number of larvae)

Table S4. List of core gene and accessory components of the type VI secretion system (T6SS) and putative function derived (30, 33, 40, 63, 72)

Gene	Homologue	Putative Function
tssI	*vgrG*	Effector/structure: forms the T6SS piercing structure
tssD	*hcp*	Effector/Structure: Homologous to T4 phage tube
tssC	*impC, vipB*	Homologous to T4 phage contractile tail sheath proteins
tssB	*impB, vipA*	Homologous to T4 phage contractile tail sheath proteins
tssH	*clpV, vasG*	ATPase /effector chaperon/recycling TssB/TssC
tssM	*vasK, icmF*	Anchoring T6SS to cell wall
tssL	*ompA, dotU*	Anchoring T6SS to cell wall
tssJ	*vasD, lip*	Anchoring T6SS to cell wall
tssE	*impF, vasS*	Essential baseplate protein similar to T4 phage gp25 proteins
tssG	*impH, vasB*	Unknown function
tssF	*impG, vasA*	Unknown function
tssA	*impA, vasJ*	Unknown function
tssK	*impJ, vasE*	Unknown function
tagB	*BB0796*	Protein with a pentapeptide_4 domain, unknown function
tagAB	*BB0795*	Protein with a pentapeptide_4 domain, unknown function
tagE	*pknA/ppkA*	Serine/threonine kinase, post-translational regulation
tagF	*impM, sciT*	Unknown function
tagG	*pppA*	Serine/threonine phosphatase, post-translational regulation
tagH	*impI*	FHA domain-containing protein, post-translational regulation
tagJ	*impE*	Unknown function
tagL	*c3389*	Protein with an OmpA C-like domain, unknown function
	VCA0105	Protein with a PAAR-motif associated with VgrG piercing structure
	L376_02862	Protein with a peptidase M_23 domain, putative endopeptidase effector
	Ebc_4130	Protein with an esterase-lipase domain, unknown function

Manuscript II

Publication status: Will be submitted to *Applied and Environmental Microbiology* in 2021.

Title: The Role of Quorum Sensing in *Vibrio coralliilyticus* RE22Sm on virulence during infection eastern oyster (*Crassostrea virginica*) larvae.

Short Title: QS mediated virulence in *V. coralliilyticus* RE22Sm

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Key Words: Quorum Sensing, Virulence, Oyster Aquaculture, *Vibrio coralliilyticus*, *Crassostrea virginica*

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Abstract

Vibrio coralliilyticus RE22 is an important pathogen in aquaculture settings and can affect many types of cultured bivalves. In larval oysters, *V. coralliilyticus* can quickly overrun larval stocks, resulting in high levels of mortality, and significant economic loss. Quorum sensing (QS) is the process by which bacteria communicate using secreted signaling molecules called autoinducers. We investigated the contributions of QS in *V. coralliilyticus* RE22Sm to the regulation of virulence factor expression and the potential role in virulence against *Crassostrea virginica* larvae. Mutations in *luxN*, *luxO*, *vcpR* and *luxM* were created and characterized for their effects on biofilm formation, extracellular metalloprotease activity, T6SS mediated killing, and virulence against *C. virginica* larvae. Based on the *Vibrio harveyi* QS system, mutations in *luxN*, *vcpR* and *luxM* were predicted to mimic a low cell density (LCD), while a mutation in *luxO* was predicted to mimic a high cell density (HCD) state. Biofilm formation was increased in the Δ*luxO* strain, while Δ*luxN*, Δ*vcpR* and Δ*luxM* mutants produced similar biofilm as the wild-type RE22Sm. Protease activity and virulence against *C. virginica* larvae were attenuated in the *luxN*, *vcpR* and *luxM* mutants. T6SS mediated killing of prey *E. coli* Sm10 was drastically attenuated in the *luxN* strain, and partially reduced in the Δ*luxO* and Δ*vcpR* strains. These data suggest that proper signal transduction via autoinducer detection by LuxN, AHL synthesis by LuxM, and transcriptional activation by VcpR is necessary for complete virulence. Further, mutation of *luxO* increased biofilm formation, suggesting a potential increase in virulence. These data are consistent with the requirement for an intact QS system for unattenuated virulence by RE22Sm against oyster larvae.

Introduction

Infectious bacterial diseases in aquatic and marine settings negatively affect development and advancement of aquaculture systems throughout the world (1, 2). *Vibrio* spp. are among the most common bacterial pathogens in marine aquaculture settings (3). *V. coralliilyticus* RE22Sm, a Gram-negative motile marine bacterium, is a member of the *Vibrionaceae* within the Gammaproteobacteria (4). Formerly classified as *Vibrio tubiashii* (5), *V. coralliilyticus* RE22 is a bacterial pathogen of larval eastern oysters (*Crassostrea virginica*) and Pacific oysters (*Crassostrea gigas*) and has been associated with major hatchery disease outbreaks, causing shortages in seed oysters for commercial shellfish producers (2, 5). Mortality rates from *V. coralliilyticus* induced vibriosis can reach 100% in both Pacific and eastern oysters and contribute to significant economic losses in aquaculture and hatchery settings (6).

Past investigations of RE22 virulence factors have focused on secreted metalloproteases (7, 8), and we have demonstrated that the two Type Six Secretion Systems (T6SS) play a significant role in virulence towards other bacteria and pathogenesis in *C. virginica* larvae (9). Additionally, the annotated genome for RE22 provides evidence for other virulence factors, including a T3SS, an RtxA-like MARTX toxin with its dedicated T1SS, a Vah1-like hemolysin (a pore-forming hemolysin), and a Plp-like hemolysin (a phospholipase) (9, 10).

Quorum sensing (QS) pathways facilitate bacterial communication by the production and sensing of diffusible signaling molecules called autoinducers. The autoinducer-1 (AI-1) pathway for intraspecies communication utilizes *N*-acyl homoserine lactones (AHLs)(11), while interspecies communication may be

accomplished by the autoinducer-2 (AI-2) pathway which relies upon furanosyl borate diester compounds (12). The AI-1 pathway in RE22Sm likely functions similarly to the well-characterized QS system in *Vibrio harveyi.* Here in *V. harveyi*, the AHL synthase, LuxM, produces the AI-1 signal, which is detected by the dual function transmembrane kinase/phosphatase LuxN. The subsequent addition or removal of a phosphate is determined by the local AHL concentration and function of LuxN. In a low cell density state (LCD), LuxN acts as a kinase to phosphorylate the QS pathway via LuxU, a phosphotransfer protein, and subsequently LuxO, a phospho-relay protein. Phosphorylated LuxO (LuxO~P) along with σ^{54} activates transcription of genes encoding five small regulatory RNAs (quorum regulatory RNA) *qrr1-5*, which bind Hfq and destabilize the mRNA of *luxR* in *V. harveyi*. This *V. harveyi* model of QS has been used to model the QS circuit in RE22Sm, but has not been demonstrated conclusively. In *V. harveyi,* the QS circuit is controlled by two master transcription factors, LuxR and AphA. LuxR controls genes under both LCD and HCD conditions, suggesting its role as the master regulator of QS. In contrast, AphA is not produced under HCD conditions, and acts to fine tune QS gene expression in the LCD state. Under low cell density conditions, T3SS and biofilm genes are up-regulated (13, 14).

In a high cell density state (HCD), the LuxN protein acts as a phosphatase to remove phosphates from the QS system when quorum levels of AHL are detected. Sufficient AHL detection triggers dephosphorylation of LuxO~P. Unphosphorylated LuxO blocks Qrr production and relieves repression of *luxR* transcription can proceed and carry out downstream QS mediated target genes (15). Under high cell density conditions, QS mediated downstream processes are up-regulated.

In *V. coralliilyticus* RE22Sm, two extracellular zinc metalloproteases, VcpA and VcpB (formerly VtpA and VtpB), are produced during the HCD state (10, 16). Work in *V. harveyi* and *V. cholerae* indicates quorum sensing controlled gene expression requires the transcriptional regulator VcpR (a LuxR homologue) (17–19). When the quorum threshold is not met, *vcpR* mRNA is not targeted for degradation by Hfq, allowing for *vcpR* transcription. Under these conditions, genes involved in T3SS (20) and biofilm formation (15, 21) are activated to facilitate further growth and AHL signal accumulation for quorum-mediated virulence. AHL production has been shown to stimulate transcription of *vcpR* (22), the master regulator of QS. Subsequent AHL detection, triggers a signal transduction cascade to trigger *vcpR* transcription, ultimately resulting in protease production by VcpAB (23).

This study examined the contributions of QS to virulence of *V. coralliilyticus* RE22Sm during oyster infection. Insertional mutagenesis was used to interrogate four QS associated genes and their impact on virulence factor production. Specifically, deletions in *luxN*, *luxO*, *vcpR* and *luxM* were created and characterized for their effects on biofilm formation, extracellular metalloprotease activity, T6SS mediated killing, and virulence against *C. virginica* larvae.

Results

The RE22Sm genome contains a *V. harveyi* type AI-1 quorum sensing system

V. coralliilyticus RE22Sm contains a functional QS circuit based on the *V. harveyi* model established by Tu *et al.* (15). The presence of a *V. harveyi* model QS system, rather than a *V. fischeri* system, was determined using readily available amino acid sequence data (4, 5). The presence of four hypothesized essential QS genes

(*luxM*, *luxN*, *luxO*, and *vcpR*) in RE22Sm were revealed by direct genome analysis via tBLASTn (Table 1). All putative QS genes in *V. coralliilyticus* shared sequence homology with the *V. harveyi* AI-1 QS system. Briefly, we examined the LuxN sequence, the dual function histidine kinase/phosphatase in *V. coralliilyticus* RE22Sm, and found certain conserved motifs that included a histidine kinase-like ATPase (HATPase) domain and a phospho-acceptor receiver (REC) domain, located at the C-terminus of the protein. The phospho-relay protein, LuxO, in *V. coralliilyticus* contains a σ^{54} interaction domain and an AtoC domain for DNA-binding transcriptional regulation. VcpR, the LuxR homolog, from *V. coralliilyticus* contains an ArcR domain for DNA-binding response regulation and an N-terminal TetR regulatory domain. The presence of a TetR domain further supports similarity to a *V. harveyi* type QS system. The AHL synthase superfamily conserved domain was detected in LuxM, the AI-1 AHL synthase. These findings indicate the presence of a *V. harveyi* AI-1 type QS system in RE22Sm.

Changes to growth and biofilm formation in quorum sensing mutants

Various *V. coralliilyticus* RE22Sm QS mutants were constructed (*luxN*, *vcpR*, *luxM*, and *luxO*), and assessed for their ability to grow planktonically or as a biofilm (Fig. 1). Under static conditions, the *luxN* mutant planktonic growth at 24 h was 0.32 log lower than wild-type RE22Sm, while the 24 h planktonic growth for the *luxO*, *luxM*, and *vcpR* mutants were 0.81, 0.83, and 0.69 log lower than wild-type RE22Sm, respectively. The *luxN* and *vcpR* mutant strain planktonic growth were not significantly different than WT RE22Sm. The *luxO* and *luxM* were significantly reduced in their planktonic growth yield when compared to WT RE22Sm (Fig. 1A). In

contrast, planktonic growth for the same strains under shaking conditions revealed that the *luxN* mutant grew to a density 0.81 log less than the wild type RE22Sm; the *luxO* mutant grew to a density 0.67 log less than RE22Sm; the *luxM* mutant grew to a density 0.39 log less than RE22Sm. In contrast, the *vcpR* mutant grew to a 0.20 log higher cell density than the wild type RE22Sm strain (Fig. 1B).

The ability of the various RE22Sm QS mutants to form a biofilm on a glass coverslip was also evaluated and compared to WT RE22Sm (Fig. 1C). Three QS mutants (*luxN*, *vcpR* and *luxM*) exhibited slightly lower (0.22 to 0.33 log decline) biofilm formation than RE22Sm wild-type cells. This difference was not significant. In contrast, the *luxO* mutant exhibited significantly greater biofilm formation (0.42 log) than WT RE22Sm. The biofilm formation ability of *luxO* mutant was significantly increased compared to the *luxN*, *vcpR*, and *luxM* mutant strains. These biofilm results strongly suggest that the amount of biofilm formation is dependent upon the state of the AI-1 QS pathway.

Changes in zinc metalloprotease activity in quorum sensing mutants

Previous studies (23–25) demonstrated the integral roles of *vcpA* and *vcpB* in metalloprotease production and activity as a virulence factor and suggested the possibility that QS regulates these genes. We examined the effect of mutations in the QS circuit on overall extracellular protease activity. Protease activity was significantly reduced in RE22Sm strains with mutations in *luxM*, *luxN*, or *vcpR* to 36%, 38%, and 37% of the activity measured in the wild type strain (Fig. 2). In contrast, Δ*luxO* strain, exhibited significantly increased protease activity (115%) compared to the wild type.

The results presented in Fig. 2 strongly suggest a direct association between extracellular metalloprotease activity and QS state.

Changes in T6SS-mediated antibacterial activity in RE22Sm Quorum Sensing Mutants

While the linkage between the T6SS and QS circuit in several other *Vibrio* species has been explored (26–30), the possible regulation of T6SS by QS has not been examined in *V. coralliilyticus*. We previously showed that RE22Sm wild-type cells kill *E. coli* Sm10 prey cells at a rate of >3 orders of magnitude over 4 h (9). We examined the ability of RE22Sm QS mutants to kill in a contact dependent manner as described in the Materials & Methods. The data shown in Fig. 3 indicate that the different QS mutations (*lux M*, *luxN*, *luxO*, and *vcpR*) each reduced T6SS-mediated killing of prey cells compared to the RE22Sm wild type strain. The wild-type RE22Sm control cells caused *E. coli* Sm10 prey cells to decline by 3.02 orders of magnitude over 4 h. In contrast, the *luxN* mutant strain exhibited the greatest loss in T6SS-mediated killing, as *E. coli* Sm10 prey cells declined only 0.90 log ($P < 0.001$) over 4 h. The *luxO* mutant strain also exhibited attenuation of T6SS-mediated killing as the *E. coli* Sm10 prey cells declined 2.35 log ($P < 0.005$) over 4 h. The *vcpR* deletion mutant was slightly attenuated and caused a prey cell decline of 2.52 log ($P < 0.005$) over 4 h. The *luxM* mutant strain showed a slight decline in its ability to kill prey cells with the *E. coli* Sm10 cell density dropping 2.71 log (n.s.) over 4 h, but this was not significantly different from the wild type. A statistical comparison of the mutants indicated that the *luxN* strain was significantly attenuated when compared to the *luxO*, *vcpR*, and *luxM* strains. There was no significant difference in virulence

between the *luxO*, *vcpR*, and *luxM* strains. These results suggest that a functional *luxN* allows for T6SS function, while *luxO* and *vcpR* attenuated virulence to a lesser degree, and *luxM* exhibited no significant effect on contact dependent virulence.

Quorum Sensing mutants predict larval oyster survival

In an effort to determine the effects of QS on oyster virulence larval oysters were challenged with RE22Sm wild type or with the QS mutant strains. Oyster larvae infected with wild type RE22Sm (at ~1×10^5 CFU/ml) for 24 h exhibited an average survival of 49.6% while the no treatment control oysters exhibited 94.7% survival. Oyster larvae were also challenged with the various QS mutant strains (Fig. 4). Knockout mutations in *luxN*, *luxM*, and *vcpR* resulted in significantly greater larval oyster survival, 85.5 %, 68.8%, and 79.3% respectively, when compared to RE22Sm wild type. In contrast, the *luxO* mutant did not exhibit enhanced oyster virulence, 48.1% survival, and showed no significant difference in larval oyster killing when compared to RE22Sm wild type.

Additionally, we tested the effects of mutations in the metalloprotease genes (*vcpA* and *vcpB*) transcriptionally regulated by VcpR upon oyster virulence (Fig. 4). Knockout mutations in *vcpA* and *vcpB* resulted in 70.6% and 72.1% oyster survival, respectively. Both survival rates are somewhat less than what was seen for the *vcpR* mutant (79.3%). No significant difference in larval oyster survival was observed when the oysters were challenged with the *vcpA*, *vcpB*, or *vcpR* mutant strains.

Discussion

The QS model described for *Vibrio harveyi* (18, 19, 31, 32) serves as a well-annotated cornerstone for QS pathway dissection in *V. coralliilyticus* RE22Sm. In certain mutants of *V. harveyi*, QS dependent bioluminescence was either removed, or constitutively expressed, based on the knockout target. For example, mutations in *luxN* (31), or *luxR* (33) eliminated bioluminescence. Regardless of true cell density in the local environment, *V. harveyi* lacking either the LuxN or LuxR proteins demonstrated a phenotype indicating insufficient cell density to change the quorum state of the organism causing the organism to be locked in a low cell density (LCD) state. Inversely, when *luxO* was knocked out, *V. harveyi* cells expressed constitutive bioluminescence (34). In this mutant background, cells perceived their local environment as a cell rich environment, with disregard for quorum regulatory molecule (AHL) concentration resulting in those cells being locked in a high cell density (HCD) state.

Based on these reported findings in *V. harveyi*, we hypothesized that since *V. coralliilyticus* RE22Sm QS contains genes that exhibit significant similarity to those in *V. harveyi* and assigned a low cell density (LCD) state designation to *luxN*, *vcpR*, and *luxM* mutants. A mutant deficient in *luxO* was hypothesized to be constitutively expressing QS mediated virulence factors and was subsequently locked into the high cell density (HCD) state. In this manuscript, we addressed the ability of the LCD and HCD mutants to function as QS mediated pathogens, and how previously described virulence factors in RE22Sm are influenced by QS state.

The results presented in this study serve to elucidate the genetic components of the AI-1 QS circuit in *V. coralliilyticus* RE22Sm, and their roles in T6SS-mediated antibacterial activity and the regulation of virulence against oyster larvae. Zhao *et al* (23) demonstrated that QS plays a role in the expression *vcpA*, *vcpB* and *vcpR* in RE22Sm. These previous findings also indicate 1000-fold increase in expression of *vcpB* over *vcpA* under standard conditions. In this study, we investigated the role of the AI-1 type QS system in RE22Sm with regards to growth, biofilm formation, protease activity, antibacterial activity, and oyster virulence. These findings serve to elucidate the roles of four genes (*luxM*, *luxN*, *luxO*, and *vcpR*) in these processes.

Based on the *V. harveyi* QS model, mutations made in certain genes (*luxN*, *vcpR*, and *luxM*) were predicted to yield a phenotype mimicking a cellular environment lacking adequate AHL concentrations. Our data suggest a density independent QS phenotype in RE22Sm strains lacking *luxN*, *vcpR*, and *luxM*. These mutant strains appear locked in a low cell density (LCD) state, regardless of actual cell density. Merodiploid insertion mutants exhibited a phenotype of reduced pathogenic potential in all experimental conditions. Of note, their reduced metalloprotease activity (Fig. 2), and attenuated larval oyster virulence (Fig. 4) support our initial hypothesis of reduced virulence in the LCD QS mutants. Despite results indicating the LCD strains had reduced planktonic growth, these findings were consistent with standard growth experiments. Nackerdien *et al.* (35) suggested the effect of quorum sensing on growth rate in *V. harveyi* can be either positive or negative, and bioluminescence tends to slow growth rate. While bioluminescence is not present in *V. coralliilyticus* RE22Sm,

other growth delaying virulence factors may be activated, or upregulated, and influence overall growth rate.

The phosphorylation state of the RE22Sm QS system was hypothesized to influence the pathogenic phenotype of RE22Sm (36). The findings presented suggest an increase in virulence in the absence of LuxO. In this cellular environment, *vcpR* transcription is activated when Qrrs are degraded in the absence of LuxO~P, thereby resulting in a potential increase in QS-mediated virulence. Zhu *et al.* (37) have demonstrated the involvement of *luxO* in *V. cholerae* virulence, yet these mutants exhibited reduced virulence in their infant mouse model by inhibiting the activity of HapR (the VcpR/TetR homolog in RE22Sm) (38). In our system, *luxO* mutations result in wild type or increased levels of virulence and do not exhibit a *vcpR* mutant phenotype. Regulation of *hapR* by *luxO* in *V. cholerae* suggests a different mechanism of regulation than in *V. coralliilyticus* RE22Sm. This may represent a future avenue of investigation in the RE22Sm QS system.

Quorum sensing detects local cell density and adjusts gene expression accordingly for a wide variety of cellular processes (39). QS can additionally regulate other cellular processes including T3SS (40, 41), T6SS (27), and biofilm formation (21, 37). We were interested in investigating the interplay between QS and the T6SS (a contact dependent system) in *V. coralliilyticus*. Ishikawa *et al.* (26) reported that QS regulates two *hcp* alleles in *V. cholerae* O1 strains, indicating *hcp* expression was growth phase dependent. HapR positively regulates expression of Hcp, while LuxO negatively regulates Hcp expression. In RE22Sm, mutants deficient in either *vcpR* or *luxO* were significantly attenuated in their T6SS killing ability, compared to the

RE22Sm WT. Further, in *V. cholerae*, Hcp expression is dependent upon the cAMP-CRP transcriptional regulatory complex and requires σ^{54} (26). Therefore, interruptions in the QS system upstream of σ^{54} involvement may control other cellular processes in RE22Sm (42). Previous studies (43) indicate QS regulation by LuxN in *V. harveyi*. Data linking LuxN to T6SS activity has yet to be characterized. These results, taken together with our findings of significantly reduced T6SS-mediated anti-bacterial and larval oyster killing activity by the *luxN* strain, strongly suggest that QS helps to regulate T6SS activity. The *luxN* mutant phenotype lacks the ability to sense and respond to the local cell density, via AHLs. The inability of LuxN in *V. harveyi* to change between kinase or phosphatase activity prohibits signal transmission along the phosphorelay system, which includes LuxO and LuxU. In our studies with RE22Sm, a mutation in *luxM*, the AI-1 AHL synthase, did not influence T6SS mediated killing of *E. coli* Sm10, and the difference in prey cell survival compared to RE22Sm wild-type was not significant. These findings suggest that QS signal production is not a driving factor for T6SS-mediated killing. Our data demonstrate that ability to sense the local environment and transmit that information, via LuxN, is more influential for RE22Sm virulence than QS signal production.

Vibriosis outbreaks have been suggested to be responsible for *C. gigas* mortality, resulting in losses of 80-100% of larvae (44). This investigation examined the roles of specific QS mutant strains and the effects these mutations have on RE22Sm virulence and larval oyster survival when challenged with the pathogen. Here, mutations in *luxN, vcpR* and *luxM* attenuate virulence, resulting in increased larval oyster survival. This may be due to decreased protease production by a defunct

QS circuit. Larval oyster survival was near identical, with no statistically significant difference between groups detected, for the *vcpA*, *vcpB*, and *vcpR* mutant strains. Survival is improved by lack of protease activity, and a previous study by Zhao *et al.* (23) demonstrated that mRNA transcription of *vcpB* and *vcpR* was inhibited by quorum quenching AHLs from the probiotic organism *Phaeobacter inhibens* S4Sm. Additionally, other groups (45–47) have described the roles of *vcpA* and *vcpB* in oyster virulence, and while it is suggested to be the major contributing virulence factor, the connection and involvement of QS in the regulation of other virulence factors, such as T6SS or hemolytic activity (48), is a target for investigation. Our findings were consistent with this study as *vcpABR* mutants improved oyster survival within the same statistical group. These data suggest an LCD state in RE22Sm attenuates virulence against larval oysters, whereas a HCD state results in wild-type levels of virulence. These data indicate that QS mediated protease activity is a secondary virulence factor in oyster infection, where the RE22Sm T6SS1 acts as the primary virulence factor (9).

Materials & Methods

Bacterial strains, plasmids and growth conditions

V. coralliilyticus RE22 strains (Table 3) were routinely cultured in yeast peptone broth plus 3% NaCl (YP30), yeast peptone broth plus 3% Instant Ocean© sea salt (mYP30), or Marine Minimal Medium (3M) plus 5% sucrose (49), supplemented with the appropriate antibiotic(s) in a shaking water bath (200 RPM) at 27°C. Overnight cultures of *V. coralliilyticus* RE22Sm, grown in mYP30, were harvested by centrifugation (8,000 × *g*; 10 min; 4˚C), and the pelleted cells washed twice with

sterile Nine Salt Solution (NSS)(24). Washed cells were resuspended to the appropriate cell densities in experimental media. *E. coli* strains were routinely cultured in LB20 (50). Antibiotics were used at the following concentrations: streptomycin, 200 µg/ml (Sm^{200}); chloramphenicol, 5 µg/ml (Cm^{5}) for *V. coralliilyticus*, and chloramphenicol, 20 µg/ml (Cm^{20}) for *E. coli*; kanamycin, 50 µg/ml (Km^{50}) for *E. coli*, kanamycin, 80 µg/ml (Km^{80}) for *V. coralliilyticus* grown in liquid media, and kanamycin 80 µg/ml (Km^{80}) for *V. coralliilyticus* grown on solid media. Agar plates were prepared using Difco Bacto© agar at 1.6%.

Characterization of *V. coralliilyticus* RE22Sm growth

A single colony of RE22Sm, or mutant strain, were inoculated into mYP30 media liquid medium, grown for 24 h at 27 ˚C with shaking, and back-diluted into 10ml fresh mYP30 at a 1:1000 dilution. Cultures were incubated at 27˚C with shaking for 24 h and aliquots were taken at select intervals (every 2 h) to determine viable bacterial counts (CFU/ml) by serial dilution and spot plating, and bacterial biomass by absorbance at 600nm. Serial dilution and OD_{600} reading were done in triplicate (n=3).

Insertional merodiploid mutagenesis

The modified pDM4 plasmid containing a kanamycin resistance (Km^{R}) gene, pDM5, was used to construct the allelic exchange mutants (Table 3) as described by Gibson *et al* (51, 52). The Km resistance gene was amplified from the TOPO2.1 vector (Invitrogen) and inserted into pDM5 via the Gibson Assembly Reaction at the AgeI restriction site. pDM5 was linearized at the SacI restriction enzyme site, using SacI-HF (New England Biolabs), within the multicloning region (MCR) for all mutation destined Gibson Assemblies. The ligation mixture was introduced into *E.*

coli Sm10 (containing λpir) by electroporation with the BioRad Gene Pulser II in a 2 mm cuvette (2.5 kV; 25 µF; 200 Ω). Transformants were selected by growth on LB20Cm^{20} agar plates, and successful mutagenesis was confirmed by PCR screening for a novel junction between the pDM4 plasmid and the Gibson Fragment(s) from *V. coralliilyticus*. The mobilizable suicide vector was transferred from *E. coli* Sm10 into *V. coralliilyticus* RE22Sm by conjugation as previously described (53). Transconjugants were selected by utilizing the kanamycin resistance (Km^{R}) gene located on the suicide plasmid. The subsequent incorporation of the target gene fragments into the suicide vector was confirmed by PCR analysis using specific primers (Table 4) to screen for the novel genetic inserts into the plasmid. The double crossover transconjugants were selected for by growth on 3MSm^{200} +5% sucrose agar plates for a second crossover event. Sucrose is used as the counter selective agent because pDM5 contains the *sacB* gene, which encodes levansucrase that converts sucrose to toxic levan (54). Putative allelic exchange mutants, and *in-cis* complements (revertants), were screened for kanamycin sensitivity. The resulting RE22Sm mutants were then screened for the desired allelic exchange double crossover using PCR amplification.

Quantification and Detection of Extracellular Metalloprotease Activity

Protease activity was quantified via the azocasein method as previously described by Denkin and Nelson (55). *V. coralliilyticus* supernatant (100 µl) was incubated for 30 minutes at 30 ^{o}C with 100 µL of azocasein solution (0.06% w/v). The reactions were terminated by adding 10%(w/v) of trichloroacetic acid (TCA) to a final concentration of 6.7% (w/v). The mixture was left undisturbed for 2 min and then

centrifuged (12,000 × *g* for 8 min) to remove residual azocasein. The supernatant containing azopeptides was suspended in 700 µL of 525 mM NaOH. Absorbance of the azopeptide containing supernatant, and negative control, was measured at 442nm [BioTek© Synergy HTX Multi-mode reader] and protease activity units (U) were calculated by:

$U = [(1{,}000 \times OD_{442})/CFU] \times 10^9$ where OD_{442} is the optical density at 442nm and CFU was calculated by serial dilution and spot plating at the beginning of each timepoint.

Biofilm Formation Assay

Biofilm formation as assessed using a modification to the crystal violet (CV) staining method (56). Bacterial strains were grown for 24 h in mYP30Sm^{200} (27°C with shaking; 200 RPM) and were diluted to ~1×10^4 CFU/ml in 5 ml of fresh mYP30Sm^{200} containing one sterile coverslip per well in a sterile 6-well tissue culture dish (untreated polystyrene) and were allowed to grow without shaking at 27 ^{o}C for 24h. Supernatant cell densities (CFU/ml) were quantified by serial dilution and spot plating on mYP30Sm^{200}. Biofilm cell density (CFU/coverslip) was quantified by removing the coverslip and washing twice in 10 ml NSS for 5 minutes. Next, the coverslip was transferred to a 50ml conical tube containing 10ml NSS and 0.5 g sterile glass beads (100 µm), and vortexed vigorously for 60 seconds.

Bacterial killing assays

Determination of T6SS-mediated killing were carried out as described by Schuttert *et al.* (9). Briefly, an attacker-to-prey ratio of 4:1 (MOI of 4), based on CFU/ml, was used. A mixture of attacker and prey cells was filtered onto a 0.22 µm filter and placed on appropriate solid growth media for 4 h. The filter was then removed from the agar plate and vortexed for 1 minute in 10 ml NSS, the culture supernatant serially diluted, and plated on appropriate differential media to enumerate the attacker cells and remaining prey cells. TCBS agar was used to select for *Vibrio spp*. and MacConkey agar to select for enteric organisms.

Larval oyster experimental challenges

Performed as previously described by Karim *et al* (57) with minor modifications. Larval eastern oysters (*Crassostrea virginica*) (6 to 10 days of age, 50 – 150 µm in size) were obtained from the Blount Shellfish Hatchery at Roger William University (Bristol, RI, USA), or Virginia Institute of Marine Science (Gloucester Point, VA, USA) or Niantic Bay Shellfish Farm (Niantic, CT, USA), and allowed to acclimate for 24 h at room temperature with gentle rocking. Approximately 100 oyster larvae were placed in each well of a 6 well plate containing 5 ml of sterilized filtered artificial seawater at 2.8% salinity. Then 50 µl of *V. coralliilyticus* RE22Sm (wild type or mutant strains) was added to the challenge wells for a final concentration of ~10^5 CFU/ml. The same volume of 2.8% ASW was added to no treatment control wells, and the plates incubated for 24 h at 20-23 ^oC with gentle nutation. Larval oysters were fed with commercial algal paste (20,000 cells/ml; Reed Mariculture Inc., San Jose, CA, USA) in order to promote ingestion of bacterial organisms. Control wells will

include non-treated larvae (with and without pathogen). Each treatment was run in triplicate and each experiment was done at least two times. Larval survival was determined 20-26 h after addition of the pathogen.

The survival rate calculated using the formula:

Survival rate (%) = 100 x (live larvae/total number of larvae)

Statistical analysis

Two-tailed Student's *t* tests assuming unequal variance were used for all statistical analyses for all detailed experiments. *P* values of < 0.05 were considered to be statistically significant.

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CS & DRN designed the study. CS created the mutant strains used in this study. CS performed all experiments under the supervision of DRN. CS and DRN wrote the manuscript with contributions and edits from DCR and MGC. Manuscript formatting was performed by CS and DRN. All authors read and approved the final version of this manuscript.

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Table 1. Sequence comparison of *V. harveyi* QS Amino Acid sequence to *V. coralliilyticus* RE22Sm genome[1]

***V. harveyi* protein**	***V. coralliilyticus* RE22Sm gene**	**Amino Acid Identity (%)**	**Amino Acid Similarity (%)**	**Inclusive Base Pairs on Chromosome 1[1]**
LuxN	*luxN*	42.48	62.00	1353531 - 1356044
LuxO	*luxO*	86.00	92.00	1648680 - 1650071
LuxR	*vcpR*	85.00	92.00	2104522 - 2105167
LuxM	*luxM*	31.39	51.00	1349349 - 1350494

[1] *V. harveyi* QS amino acid sequences [query] compared to *V. coralliilyticus* RE22Sm genome [subject] by tBLASTn using default parameters. (40)

Table 2. Bacterial strains and plasmids used in this study

Strain	Description	Resistance(s)	Reference
V. coralliilyticus			
RE22	Wild-type isolate from oyster larvae		Ester et al, 2004
RE22Sm	Spontaneous Sm^r mutant of RE22	Sm^r	Zhao et al, 2016
RE22SmKm	Sm^r Km^r mutant of RE22 harboring an empty pSUP203 shuttle vector	Sm^r Km^r	This Study
RE22 *luxN*	Sm^r Km^r; insertional merodiploid mutation of *luxN* using pDM5	Sm^r Km^r	This Study
RE22 *luxO*	Sm^r Km^r; insertional merodiploid mutation of *luxO* using pDM5	Sm^r Km^r	This Study
RE22 *vcpA*	Sm^r Km^r; insertional deletion mutation of *vcpA* using pDM4	Sm^r Cm^r	This Study
RE22 *vcpB*	Sm^r Km^r; insertional deletion mutation of *vcpB* using pDM4	Sm^r Cm^r	This Study
RE22 *vcpR*	Sm^r Km^r; insertional merodiploid mutation	Sm^r Km^r	This Study

	of *vcpR* using pDM5		
RE22Δ*luxM*	Sm^r Km^r; insertional merodiploid mutation of *luxM* using pDM5	Sm^r Km^r	This Study
E. coli			
Sm10	*Thi thr leu tonA lacY supE recA* RP4-2 Tc::Mu::Km (λ)	Km^r	Simon et al, 1983
Sm100	Sm10 harboring pDM5 plasmid	Km^r Cm^r	This Study
S122	Sm10 harboring pSUP202P-*gfp*(ORF)	Km^r	Zhao et al, 2016
CS11	Sm10 harboring pDM5-*luxN*	Km^r Cm^r	This Study
CS12	Sm10 harboring pDM5-*luxO*	Km^r Cm^r	This Study
CS13	Sm10 harboring pDM5-*vcpA*	Km^r Cm^r	This Study
CS14	Sm10 harboring pDM5-*vcpB*	Km^r Cm^r	This Study
CS15	Sm10 harboring pDM5-*vcpR*	Km^r Cm^r	This Study
CS16	Sm10 harboring pDM5-*luxM*	Km^r Cm^r	This Study
Plasmids			

pDM4	Cm^r; suicide vector with R6K origin and *sacB*	Cm^r	Milton et al, 1996
pDM5	Cm^r Km^r; suicide vector with R6K origin and *sacB*	Cm^r Km^r	This Study
pSUP202P	Ap^r Cm^r Tc^r; broad host shuttle vector	Ap^r Cm^r Tc^r	Simon et al, 1983
pSUP203	Ap^r Cm^r Tc^r Km^r; broad host shuttle vector	Ap^r Cm^r Tc^r Km^r	This Study

Table 3. Primers used in this study

Primer	**Sequence (5' to 3', underlined sequences are engineered for Gibson Assembly sites in pDM5)**	**Description**
PmN7	tgtggaatcccgggagagctAACAGTTATTTTCATACAGATCTTC	For *luxN* insertional mutation, 5' forward
PmN8	ctcttattgtAAATCATGTAAATTACACGAGTTC	For *luxN* insertional mutation, 5' reverse
PmN9	tacatgatttACAATAAGAGTGGCGGCAG	For *luxN* insertional mutation, 3' forward
PmN10	gcatgcgggtaacctgagctTTCTCCCGATTCACCTGATAG	For *luxN* insertional mutation, 3' reverse
PmO1	aatcccgggagagctCCGATAAAGCCTTGATAATTCTGGTTACC	For *luxO* insertional mutation, 5' forward
PmO2	gaagggaaTTGAAGATACTGCGTCGGTCG	For *luxO* insertional

		mutation, 5’ reverse
PmO3	gtatcttcaaTTCCCTTCACAGGCCTGAATC	For *luxO* insertional mutation, 3’ forward
PmO4	cgggtaacctgagctTGCAAACTAAGCTACTACGTTTTATTCAGAC	For *luxO* insertional mutation, 3’ reverse
PmA53	tgtggaatcccgggagagctTACCAGTTACAGCCGCAG	For *vcpA* insertional mutation, 5’ forward
PmA54	acctgaagtaCAACAAAAAAGTCTACCATGTAAAC	For *vcpA* insertional mutation, 5’ reverse
PmA55	ttttttgttgTACTTCAGGTAGCACAGC	For *vcpA* insertional mutation, 3’ forward
PmA56	gcatgcgggtaacctgagctTAGAAGGCACGGTTGTAC	For *vcpA* insertional mutation, 3’

		reverse
PmB57	tgtggaatcccgggagagctTTCTGAGTAACCGAATACGTTGAC	For *vcpB* insertional mutation, 5' forward
PmB58	tgactgtggaGCACGAAGTCAGCCATGG	For *vcpB* insertional mutation, 5' reverse
PmB59	gacttcgtgcTCCACAGTCACAACGTTATC	For *vcpB* insertional mutation, 3' forward
PmB60	gcatgcgggtaacctgagctTCTGGGCTGAATTCTCAG	For *vcpB* insertional mutation, 3' reverse
PmR11	tgtggaatcccgggagagctTATACAACTCAATTGGCAAGG	For *vcpR* insertional mutation, 5' forward
PmR12	gattgctttgATGCGATTTCCATCAGTTG	For *vcpR* insertional mutation, 5' reverse

PmR13	gaaatcgcatCAAAGCAATCGAGCGTGG	For *vcpR* insertional mutation, 3' forward
PmR14	gcatgcgggtaacctgagctTCTAGGTAGCTTTGTGTCAG	For *vcpR* insertional mutation, 3' reverse
PmMfp	GCCGAGCTCCACAACATGCAGAAATACTC	For *luxM* insertional mutation, forward
PmMrp	GCCTCTAGAAACTCAATTTATGGCGTTCT	For *luxM* insertional mutation, reverse

Figure 1. Quantification of *V. coralliilyticus* RE22Sm QS mutants during (A) planktonic growth (CFU/ml), (B) shaking growth (CFU/ml), and (C) biofilm formation (CFU/coverslip). (D) planktonic growth of *in-cis* complements, and (E) biofilm formation of *in-cis* mutant complements. Cultures of each strain were grown at 27°C for 24 h. The data are the average of 3 biological replicates (experiments); each experiment had three technical replicates. Error bars represent ±1 standard deviation (SD). Statistical analysis by unpaired Student's T-test were made against RE22Sm wild-type. ns = not significant, * = $P < 0.05$. (Static growth in 6-well plates with 5 ml/well)

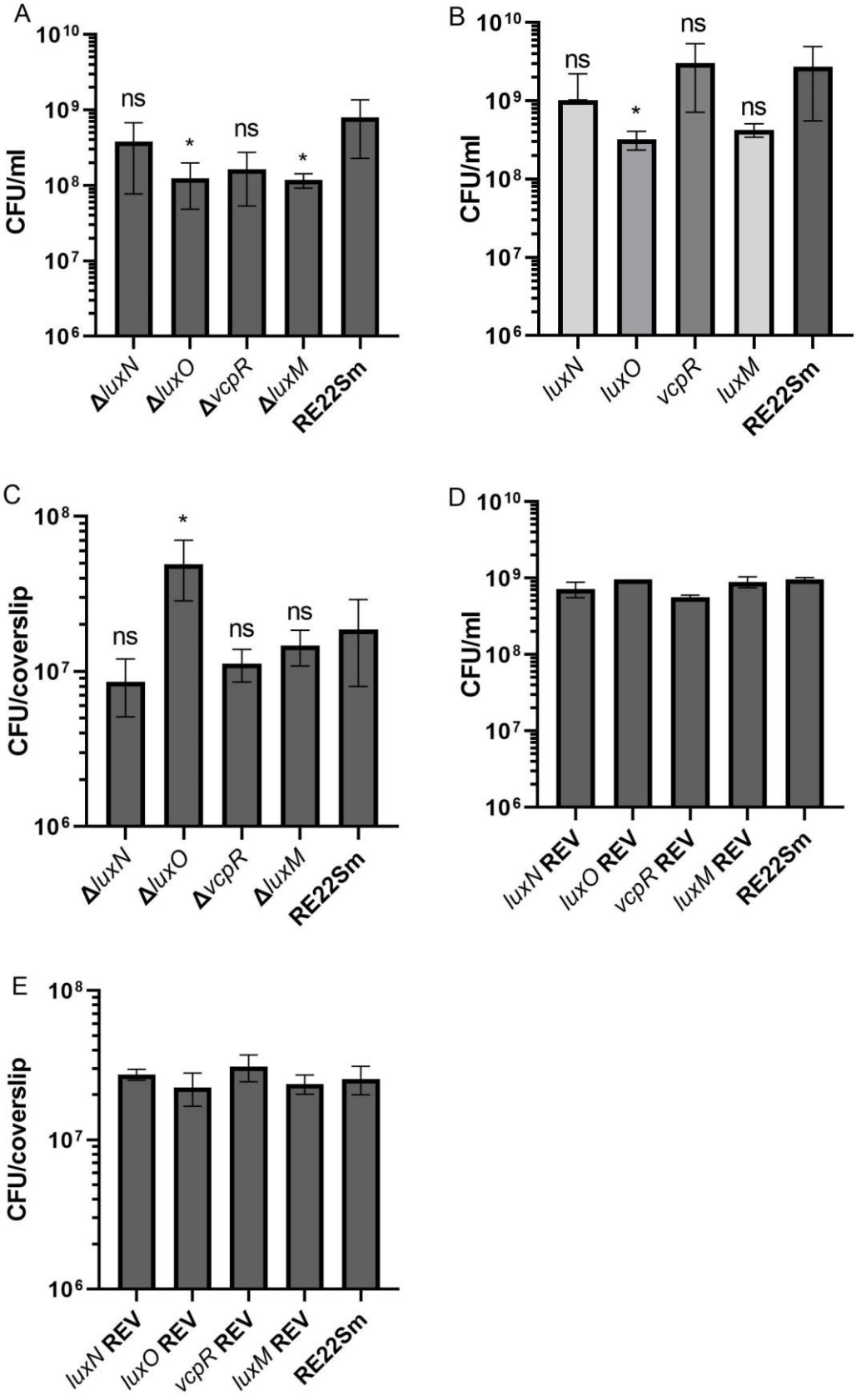

A
CFU/ml
ns
*
ns
*
ΔluxN
ΔluxO
ΔvcpR
ΔluxM
RE22Sm
B
CFU/ml
ns
*
ns
ns
luxN
luxO
vcpR
luxM
RE22Sm
C
CFU/coverslip
ns
*
ns
ns
ΔluxN
ΔluxO
ΔvcpR
ΔluxM
RE22Sm
D
CFU/ml
luxN REV
luxO REV
vcpR REV
luxM REV
RE22Sm
E
CFU/coverslip
luxN REV
luxO REV
vcpR REV
luxM REV
RE22Sm

Figure 2. Determination of *V. coralliilyticus* RE22Sm QS mutant strains extracellular metalloprotease production over 4 h (*luxN* = circle, *luxO* = square, *vcpR* = triangle, *luxM* = diamond, RE22Sm = ×). All strains were washed twice in NSS and resuspended to ~1×10^9 CFU/ml. Supernatant from each strain was boiled at 100 ^oC and used as a negative control and blank. The data are the average of 3 biological replicates (experiments); each experiment had three technical replicates. Error bars represent ±1 standard deviation (SD). Statistical analysis by unpaired Student's T-test, compared to RE22Sm wild-type. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$.

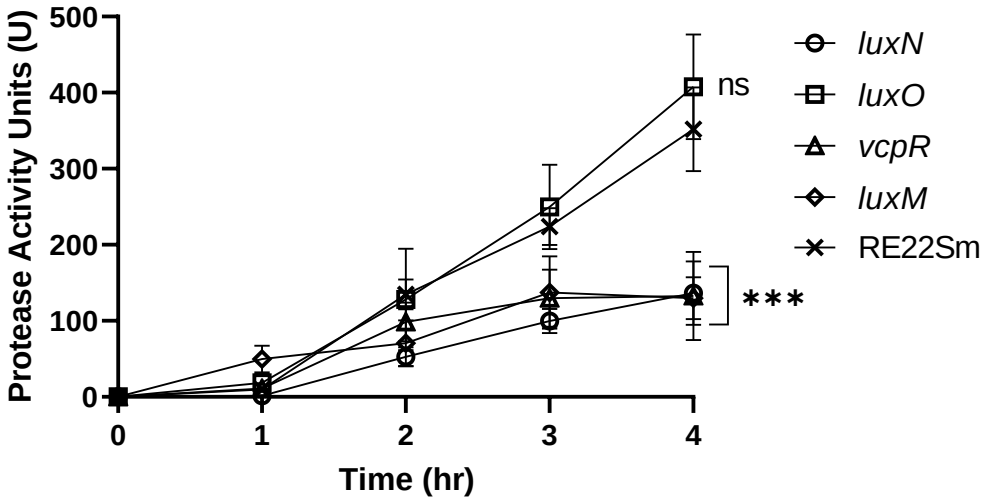

Protease Activity Units (U)
500
400
300
200
100
0
0
1
2
3
4
Time (hr)
ns
***
luxN
luxO
vcpR
luxM
RE22Sm

Figure 3. Determination of the *V. coralliilyticus* RE22Sm wild type and QS mutant strains (attacking cells) T6SS-mediated antibacterial activity against *E. coli* Sm10 (prey cell) when incubated on a filter for 4 h at 27°C with a 4:1 predator: prey ratio (MOI = 4). Starting RE22 cell density was ~2×10^9CFU/ml and starting *E. coli* Sm10 cell density was ~5×10^8 CFU/ml. The data are the average of 3 biological replicates (experiments); each experiment had three technical replicates. Error bars represent ±1 standard deviation (SD). Statistical analysis by unpaired Student's T-test, and compared to RE22Sm wild-type. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$.

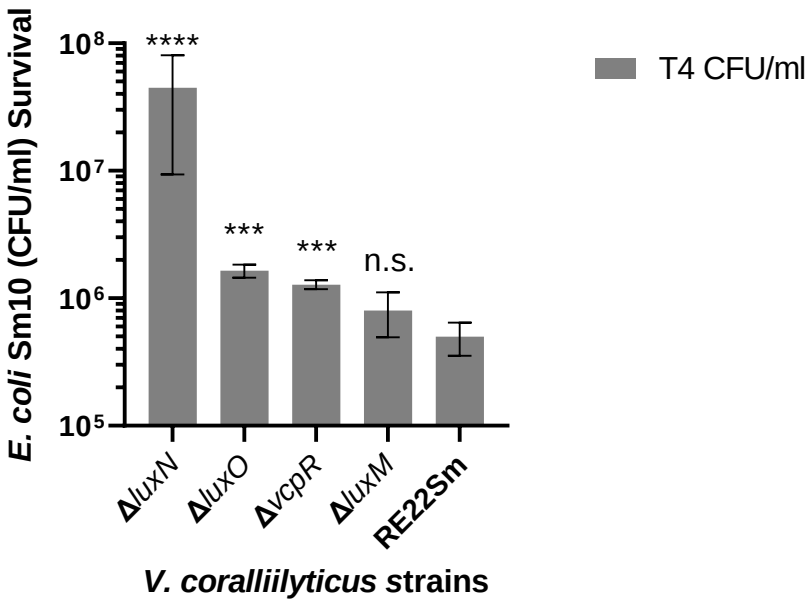
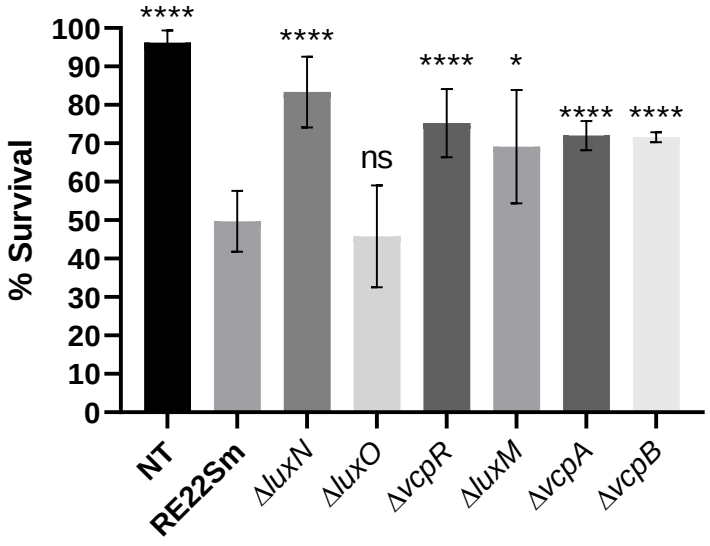

E. coli Sm10 (CFU/ml) Survival
10^8
10^7
10^6
10^5
****
***
***
n.s.
ΔluxN
ΔluxO
ΔvqpR
ΔluxM
RE22Sm
V. coralliilyticus strains
T4 CFU/ml

Figure 4. Oyster larvae survival after challenge *with V. coralliilyticus* RE22Sm. Oyster larvae were exposed to RE22Sm wild type and mutant strains (1×10^5 CFU/ml) for 24 h. Oyster larvae treated with artificial seawater served as the negative control. Larval survival (% ±1 SD) was determined once RE22Sm wild type killing reached 40-60%. The data are the average of 3 biological replicates (experiments); each experiment had three technical replicates. Error bars represent ±1 standard deviation (SD). Statistical analysis by unpaired Student's T-test, and compared to RE22Sm wild-type. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$.


% Survival
100
90
80
70
60
50
40
30
20
10
0
****
****
****
ns
*
****
****
NT
RE22Sm
ΔluxN
ΔluxO
ΔvcpR
ΔluxM
ΔvcpA
ΔvcpB

Manuscript III

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Title: The Role of Quorum Sensing in *Phaeobacter inhibens* S4Sm and Effects on Probiotic Activity.

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Key Words: *Phaeobacter inhibens*, *N*-acyl homoserine lactones, quorum sensing, TDA, probiotic, *Vibrio coralliilyticus*, oyster disease

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Abstract

The gram-negative probiotic bacterium *Phaeobacter inhibens* strain S4Sm was isolated from the inner shell surface of a healthy oyster, secretes the broad-spectrum antibiotic tropodithietic acid (TDA), synthesizes inhibitory *N*-acyl homoserine lactones (AHLs), forms robust biofilms, and increases larval oyster survival when challenged with bacterial pathogens. Here, we investigated the roles of quorum sensing (QS) genes in probiotic activity. Mutations in *pgaI* (AHL synthase), *pgaR* (cognate AHL receptor and transcriptional regulator), *luxO* (phosphorelay protein), and *pgaK* (transmembrane histidine kinase/phosphate) were generated by insertional mutagenesis by homologous recombination. Mutation of either *pgaI* or *pgaR* resulted in the loss of TDA production, a greater than 100-fold decline in biofilm formation, and the overall loss of probiotic activity. Mutation of *luxO* or *pgaK* resulted in increased amounts of C_{14}-AHL production, small (but not significant) increases in biofilm formation, and a small increase in probiotic activity. These findings indicate that the probiotic activity of *P. inhibens* S4Sm is strongly influenced by AHL production/detection. Targeted exploitation of the QS system by mutagenesis, of the *luxO* and *pgaK* genes, increased probiotic activity and larval oyster survival when challenged with *V. coralliilyticus* RE22.

Introduction

Marine pathogens pose an imminent threat to global aquaculture infrastructure, quickly causing severe disease and high mortality. If uncontrolled, infection directly correlates to significant economic losses, and interruption of aquaculture product availability (1, 2). Larval and juvenile aquaculture species are particularly sensitive to pathogenic attack. Most marine bivalve products are harvested from an aquaculture setting, and valued at over 20 billion USD per year worldwide (3, 4). Opportunistic pathogens within the Vibrionaceae family frequently cause disease in a variety of shellfish (5, 6). For example, *Vibrio coralliilyticus* RE22 (reclassified from *V. tubiashii* RE22 (7–9)), a pathogen targeting larval bivalve species has been responsible for large scale mortalities among bivalve species on the western coast of the United States (10). Vibriosis can cause decreased larval motility and tissue necrosis within 24 h of exposure to the pathogen.

V. coralliilyticus RE22 contains a suite of virulence factors, including, but not limited to, hemolysins, extracellular metalloproteases, and two type six secretion systems (T6SS), and are found to be involved in bacterial antagonism and larval oyster virulence. *V. coralliilyticus* RE22 harbors two metalloproteases, VcpA and VcpB (formerly VtpA & VtpB), at least one hemolytic gene locus *vchAB*, and two T6SSs with differential, and overlapping, prey specificity (6, 11, 12). While the regulation of protease activity is not fully understood, other groups (13) have reported that VcpR, a TetR type transcriptional regulator, positively regulates many virulence factor genes, including *vcpA*, *vcpB*, *aphA*, and *vchAB* in *V. coralliilyticus* (13–15). Additionally, VcpR shares homology to other quorum sensing (QS) regulators including the

orthologs LuxR (from *V. harveyi*; 84% identity), and HapR (from *V. cholerae*; 75% identity). These findings suggest that VcpR functions as a QS regulator, however the precise mechanism has yet to be defined. Findings by Schuttert et al (12) demonstrate that mutation of *vcpR* significantly reduces *V. coralliilyticus* virulence factor production and improves larval oyster survival.

Phaeobacter inhibens S4Sm, a member of the *Roseobacter* clade within the *Alphaproteobacteria*, was isolated from the inner shell surface of a healthy oyster (16). It exhibits reliable probiotic activity in the protection of larval oysters from *V. coralliilyticus* RE22 and *Alliiroseovarius crassostreae* infection (16). Other investigators have also shown the probiotic potential of other strains of *P. inhibens* in protecting cod larvae (17) and other fish against pathogenic vibrios (18). Previous works have investigated the probiotic mechanisms of *P. inhibens*. The broad spectrum ionophore antibiotic tropodithietic acid (TDA), produced by this probiont, has been shown to protect finfish (18, 19) and shellfish (16, 20) from vibrio challenge. Acquired resistance to TDA is difficult to acquire and maintain (21). *P. inhibens* S4Sm also produces robust biofilms to colonize and protect host organisms (16, 21). *P. inhibens* also produces *N*-acyl homoserine lactones for quorum sensing. These AHL molecules serve as part of a global cellular communication system, competitively inhibit *V. coralliilyticus* QS molecules (22), and act in a positive feedback loop with TDA (23). These characteristics function together to enable *P. inhibens* strains to act as a robust probionts.

We sought to understand the interplay between QS genes, the ability to withstand T6SS-mediated antibacterial activity of *V. coralliilyticus*, and the probiotic activity of

P. inhibens S4Sm to protect oyster larvae. Here, we report exploitation of the QS system by mutagenesis of four involved genes, and the implications of these gene products on *P. inhibens* growth, biofilm formation, TDA production, susceptibility to type six secretion mediated attack, and the ability of these mutant strains to protect *C. virginica* larvae. These findings indicate that probiotic mechanisms in *P. inhibens* S4Sm are QS dependent.

Results

P. inhibens mutant strains *pgaI* and *pgaR* lack C_{10} and C_{14} AHL production

P. inhibens S4Sm wild-type and mutant strains were cultured and AHLs were extracted as described in Materials & Methods. LCMS/MS analysis was then used to detect and measure the production of AHLs in *P. inhibens* S4Sm wild-type (WT) and mutant strains. The ten carbon (C_{10}) AHL, (3*R*)-*N*-(3-hydroxydecanoyl)-L-homoserine lactone, was detected in *P. inhibens* S4Sm wild-type supernatant at an intensity of 1.15×10^4 counts per second (cps) (Fig. 1A). In contrast, the C_{10} AHL was detected only in trace amounts (< 0.55% of WT) in the *pgaI* and *pgaR* mutants. The *luxO* and *pgaK* mutants produced the C_{10} AHL at only 40% and 60% of the wild-type S4Sm levels, respectively. The fourteen carbon (C_{14}) AHL, (3*R*,7*Z*)-*N*-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone (Fig. 1B) was produced by *P. inhibens* S4Sm wild-type, but not by either the *pgaI* or *pgaR* mutants and was greatly diminished in the in *luxO* mutant supernatant. In contrast, production of the long chain C_{14} AHL by the *pgaK* mutant was increased 2.29-fold compared to wild-type S4Sm levels. The previously described C_{12} AHL (22) was not detected in *P.*

inhibens S4Sm wild-type or mutant culture supernatants under the culture conditions used.

Quorum Sensing Mutations *pgaI* & *pgaR* in *P. inhibens* S4Sm Reduce Cell Growth

P. inhibens S4Sm wild-type and QS mutants were grown under shaking conditions as described in the Materials & Methods. The QS deficient mutants *pgaI* and *pgaR* grew to a significantly lower cell density of 2.60×10^8 CFU/ml and 3.90×10^8 CFU/ml, respectively, at 24h, when compared to S4Sm WT (8.33×10^8 CFU/ml). The two other QS mutant strains, *pgaK* and *luxO* (Fig. 2) exhibited more robust growth at 24 h reaching final densities of 7.56×10^8 CFU/ml and 6.06×10^8 CFU/ml, respectively at 24 h, which were not significantly different from S4Sm WT.

AHL production affects Planktonic Growth and Biofilm Formation Ability in *P. inhibens* S4Sm

We examined the planktonic growth and biofilm formation ability of *P. inhibens* S4Sm in a standard biofilm formation assay (described in Materials and Methods). *P. inhibens* S4Sm grown under static conditions (Fig. 3A) demonstrated increased planktonic cell density for the *pgaK* (14.3-fold increase) and *luxO* (6-fold increase) mutant strains, when compared to S4Sm WT (4.08×10^7 CFU/ml). In contrast, the *pgaI* (9.17×10^6 CFU/ml) and *pgaR* (5.35×10^6 CFU/ml) mutant strains exhibited significantly decreased planktonic growth (22% and 13%, respectively) compared to S4Sm WT.

Biofilm formation ability (Fig. 3B) results suggested *pgaK* (4.9-fold increase) formed a more robust biofilm than wild-type S4Sm (1.07×10^6 CFU/coverslip). In this

assay, the *luxO* (1.38×10^6 CFU/coverslip) mutant formed biofilm at about the same cell density as S4Sm WT. Further, mutants deficient in *pgaI* (0.004-fold of WT) and *pgaR* (0.0033-fold of WT) had significantly reduced biofilm ability when compared to wild type *P. inhibens* S4Sm.

P. inhibens S4Sm Inhibition of *Vibrio coralliilyticus* and *Vibrio anguillarum* growth is QS dependent

It has been previously shown that TDA production in *P. inhibens* is regulated by QS (24). We examined the ability of *P. inhibens* S4Sm QS mutants and the ability to inhibit coral and oyster pathogen *V. coralliilyticus* RE22Sm and *Vibrio anguillarum* NB10Sm, a pathogen of finfish, crustaceans and bivalves, as described in the Materials & Methods section. Both the *pgaI* and *pgaR* mutant strains failed to inhibit *V. coralliilyticus* RE22Sm growth with no measurable zone of inhibition (ZOI) detected (Fig. 4A). The *luxO* mutant inhibited RE22Sm growth, and demonstrated a 0.70 mm ZOI, and was not significantly different from the WT S4Sm ZOI (0.50 mm). The *pgaK* mutant caused a 1.10 mm ZOI against RE22Sm, which was a significantly greater zone of inhibition than that of WT S4Sm. When tested against *V. anguillarum* NB10Sm (Fig. 4B) both the *pgaI* and *pgaR* mutants failed to create inhibitory zones against NB10Sm. In contrast, both the *luxO* and *pgaK* mutant strains did produce a ZOI against NB10Sm, measuring at 2.10 mm and 2.75 mm, respectively, but were not significantly different from S4Sm wild-type ZOI of 1.80 mm. Additionally, the ZOIs formed by *luxO*, *pgaK*, and WT S4Sm against NB10Sm were significantly larger than ZOIs produced against RE22Sm.

TDA and AHL production are necessary for resilience against T6SS mediated attack by *V. coralliilyticus* RE22Sm

In order to assess the susceptibility of S4Sm to a contact mediated T6SS attack, we utilized a modified T6SS assay protocol as previously described by Schuttert *et al.* (12) with minor modifications. Using *V. coralliilyticus* RE22Sm as the attacking cells, over the course of 4 h, S4Sm WT cells declined 0.86 log (from 4.21×10^8 CFU/ml to 5.78×10^7 CFU/ml). The S4Sm *pgaI* mutant cells exhibited greater sensitivity to attack and declined 2.50 log over 4 h (from 4.85×10^8 CFU/ml to 1.52×10^6 CFU/ml). The *pgaR* mutant cells also showed increased sensitivity and declined 2.44 log (from 5.2×10^8 CFU/ml to 1.87×10^6 CFU/ml) over 4 h. The *luxO* mutant cells declined 1.30 log (from 5×10^8 CFU/ml to 2.53×10^7 CFU/ml) over 4 h. The *pgaK* mutant declined 1.37 log (from 6×10^8 CFU/ml to 2.53×10^7 CFU/ml) over 4 h. The *clpX* mutant (25) declined 1.83 log (from 5.22×10^8 CFU/ml to 7.67×10^6 CFU/ml) over 4 h. The *tdbD* mutant, (25, 26), declined 2.51 log over 4 h from 6.85×10^8 CFU/ml to 2.1×10^6 CFU/ml. The *clpX* and *tdbD* mutant strains are deficient in TDA biosynthesis, and were not significantly different in their survival after 4 h. The *exoP* mutant (16, 25) declined 0.72 log over 4 h from 7.8×10^8 CFU/ml to 1.48×10^8 CFU/ml, and had no effect on survival in the T6SS assay compare to the WT S4Sm cells (Fig. 5). These data suggest that for *P. inhibens* S4Sm requires both TDA biosynthesis and AHL production to remain resilient against T6SS pathogenic activity by *V. coralliilyticus* RE22Sm.

Effects of *P. inhibens* S4 QS mutations on probiotic activity against *V. coralliilyticus* RE22 in oyster larvae

In order to determine if QS mutations would affect *P. inhibens* S4Sm probiotic activity against *V. coralliilyticus* RE22Sm *in vivo*, larval oyster challenge assays were performed as described by Karim et al (16). *P. inhibens* S4Sm QS mutants (*pgaI* & *pgaR*) showed a significant reduction in protective ability. The *luxO* and *pgaK* mutant strains showed no change or increased protection, respectively, as compared to the S4 WT (Fig. 6). Mutations in *pgaI* (76% ± 2.8% survival) and *pgaR* (77% ± 2.8% survival) showed a significant decline in the ability to protect oyster larvae against *V. coralliilyticus* challenge compared to S4 WT (87% ± 3.46% survival). The *luxO* (84% ± 6.5% survival) mutant showed no difference in protective ability compared to S4 WT. The *pgaK* (93% ± 2.4% survival) mutant strain significantly increased larval survival compared to S4Sm WT. Further, there was no significant difference between oysters pretreated with the *pgaK* mutant strain, and challenged with *V. coralliilyticus*, and the no treatment (NT) control. Larvae challenged with *V. coralliilyticus* alone were used as a control and were counted when survival ranged between 40-60% (mean = 52% ± 6.1%, median = 52%).

Discussion

In this study, we present data that *P. inhibens* S4Sm production of AHLs, TDA biosynthesis, and biofilm formation are under the control of quorum sensing. Quorum sensing has been implicated in regulation of many physiological functions, in both gram-negative and gram-positive organisms, including virulence, symbiosis, motility, conjugation, antibiotic production, and biofilm formation (27). These QS controlled probiotic functions may contribute to inhibition of virulence factors produced by the marine pathogen *Vibrio coralliilyticus* RE22Sm. TDA also actively kills *Vibrio* sp. (28). Specifically, we verified that mutations in *pgaI*, the gene responsible for AI-1 AHL synthesis, and *pgaR*, the cognate AHL signal receptor, blocked production of the C_{10} (Fig. 1A) and C_{14} (Fig. 1B) AHLs. In contrast, the *pgaK* mutant produced over two-fold higher amounts of the long chain C_{14} AHL than did the wild-type S4Sm.

The overproduction of the C_{14} AHL by *pgaK* may introduce new QS mediated effects, as Zhao et al (22) indicated the C_{14} AHL to be a less potent QS inhibitor of *V. coralliilyticus vcpR* transcription and, therefore, metalloprotease activity. The reported findings in the *pgaK* mutant may more strongly influence the positive feedback loop of AHL production and TDA biosynthesis. These findings indicate a potential avenue for future studies on the contributions of the C_{14} AHL on probiotic function in *P. inhibens* S4Sm. Previous findings by Zhao et al. (22) reported the production of an additional, twelve carbon, AHL. This AHL was not detected in culture supernatant under these conditions.

Probiotic phenotypes in the *pgaI* and *pgaR* strains was universally reduced. Additionally, when mutations were generated in *luxO* (phosphorelay protein) and

pgaK (histidine kinase/phosphatase), probiotic activity was consistent with S4Sm WT or increased overall, respectively. Growth data for these strains indicate wild type levels of growth (Fig. 2A & 2B), eliminating the possibility that the phenotypes illustrated in this study were density dependent. Our data suggest that *P. inhibens* S4Sm probiotic function is related to its QS state, and deletion of certain components of the regulatory system can decrease or increase the probiotic activity of *P. inhibens* S4Sm to prevent oyster infection by *V. coralliilyticus* RE22Sm.

Quorum sensing has been implicated in regulation of many physiological functions, in both Gram negative and Gram positive organisms, including virulence, symbiosis, motility, conjugation, antibiotic production, and biofilm formation (27). Gram-negative organisms, like *P. inhibens* S4Sm, typically use AHLs as autoinducers for QS. AHLs can also function as quorum quenching (QQ) molecules by disrupting other QS pathways and bacterial physiological functions (29). In this case, *P. inhibens* S4Sm (22) AHLs were previously shown to repress protease activity in *V. coralliilyticus* RE22Sm by blocking *vcpR* transcription; VcpR is the transcriptional activator of the *vcpA* and *vcpB* protease genes. QQ AHLs produced by S4Sm have been shown to decrease protease production in RE22Sm (22), perhaps by binding more competitively to LuxN than RE22Sm QS AHLs. The *vcpR* gene is a *luxR* homolog and part of the QS signal transduction pathway (30). The VcpA and VcpB proteases were previously identified as major virulence factors in this marine pathogen (6). Quorum quenching (QQ), through physical constraint or enzymatic degradation, activity has been described in many prokaryotes (31).

We speculate that AHLs produced by *P. inhibens* S4Sm may function to block T6SS activity in RE22Sm. QQ may act directly on *vcpR*, or similar transcriptional activators. T6SS activity was drastically attenuated by insertional deletion mutagenesis of *luxN* (AI-1 kinase/phosphatase sensor) in *V. coralliilyticus* RE22Sm (Schuttert, in preparation). QQ AHLs produced by S4Sm have been shown to decrease protease production in RE22Sm (22), perhaps by binding more competitively to LuxN than RE22Sm QS AHLs. Additionally, TDA has been shown to collapse the proton motive force (PMF) in susceptible bacteria, and could result in failed firing of the T6SS.

The protective effects of the S4Sm mutants were strain dependent in all experiments. Biofilm formation and planktonic cell growth (in static culture) (Fig. 3) were significantly reduced in the *pgaI* and *pgaR* strains when compared to wild-type biofilm formation and planktonic growth. In contrast, the *luxO* mutant, which was hypothesized to mimic a high cell density (HCD) environment, formed biofilms and grew to a cell density similar to S4Sm WT. Due to *luxO* mutation-induced HCD and that pretreatment with S4Sm vastly improves oyster larvae survival (16), we predicted greater probiotic function in the *luxO* mutant strain. The *pgaK* mutant strain formed a more robust biofilm and grew to a higher planktonic cell density than wild type S4Sm. PgaK in S4Sm shares conserved domains with the LuxN AI-1 AHL receptor in RE22Sm. This similarity would indicate AHL recognition function, and may be involved positive feedback loop of AHL production and TDA biosynthesis in S4Sm (32). These findings suggest that the ability of *P. inhibens* S4Sm to synthesize or recognize QS molecules is essential to probiotic function against *V. coralliilyticus*

RE22Sm. One possibility for this observation is the absence of pigmentation of the *pgaI* and *pgaR* S4Sm strains, as TDA is a yellow compound (23) and has been previously described to function in a positive feedback loop in conjunction with AHLs (24, 33), potentially functioning as a quorum-controlled self-enhancing molecule (34).

TDA is a broad-spectrum antibiotic created by marine bacteria, including *P. inhibens* S4Sm. TDA functions similarly to polyether antibiotics, and collapses the proton motive force of susceptible bacteria (21). Previous studies have indicated that AHL production and TDA biosynthesis exist in a positive feedback loop (32). Based on these data, we predicted that QS deficient mutants, *pgaI* and *pgaR*, would lack ZOI capabilities, whereas QS enhanced mutants, *luxO* and *pgaK*, would exhibit increased ZOI against *Vibrio* sp. Here, mutants deficient in QS signaling lack the characteristic yellow phenotype, and exhibit no ZOI against either RE22Sm or the more sensitive NB10Sm strain. Mutants capable of AHL production (*luxO* and *pgaK*) were able to inhibit RE22Sm and both strains exhibited no statistical difference in ZOI from S4Sm WT in the NB10Sm experiment. While not statistically different, both *luxO* and *pgaK* strains produced larger ZOIs under these conditions. These findings are consistent with previously reported finding by Zhao et al. (25), where *V. anguillarum* NB10Sm were found to be more sensitive to inhibition by TDA than *V. coralliilyticus* RE22Sm.These findings suggest that AHL production is vital for inhibition of the marine pathogens *V. coralliilyticus* RE22Sm and *V. anguillarum* NB10Sm.

The T6SS is a contact dependent virulence mechanism with differential target specificity employed by *V. coralliilyticus* RE22Sm (12). The RE22Sm genome contains two T6SSs with different, yet complementary, target activity for bacterial

antagonism and virulence against larval oysters. Previous data suggests that both T6SSs are required for full virulence, and may act together in the oyster system (12). Where, antagonism by T6SS2 may clear a niche in the *C. virginica* microbiome for RE22Sm colonization, followed by T6SS1 attack directly on larval larvae. The T6SS assay allows for the study of the close-quarters interactions between RE22Sm and S4Sm, and an investigation of how these organisms interact in the larval oyster model. The ability of RE22Sm to attack and kill *P. inhibens* was of interest. Our results indicate that *P. inhibens* strains lacking the ability to produce AI-1 AHLs (*pgaI*), synthesize TDA (*pgaI, pgaR, clpX, tdbD*), or form biofilms at wild-type levels (*clpX*) negatively impacted S4Sm resilience against T6SS mediated attack by *V. coralliilyticus* RE22Sm. Mutant strains deficient in one or more probiotic mechanisms were significantly more vulnerable to the RE22Sm T6SS. S4Sm mutants hypothesized to have increased probiotic potential (*luxO* & *pgaK*) were more resilient to T6SS mediated attack, and were akin to S4Sm wild-type levels of survival under these conditions.

In order to function as a true probiont, a 24 h pretreatment with S4Sm is essential. Previous studies (16) (25) indicate that when added at the same time S4Sm is outcompeted by RE22Sm in both the larval oyster challenge system and during a biofilm formation assay. However, when pretreated with S4Sm 24 h prior to introduction of the pathogen, S4Sm is able to colonize the environment, reducing or preventing RE22Sm mediated mortality of oyster larvae, and colonize glass coverslips to reduce the ability of RE22Sm to colonize the glass coverslip. Here, when oyster larvae were pretreated with S4Sm wild type or QS mutants, larval survival increased

in TDA producing QS mutants. QS deficient mutants *pgaI* and *pgaR* were unable to protect oyster larvae, when challenged with RE22Sm. The *luxO* mutant, which mimics a constitutive quorum state, slightly, but not significantly, increased larval survival to S4Sm wild-type levels (90% survival). Interestingly, the *pgaK* mutant, which lacks a *walK*-like dual function histidine kinase/phosphatase cytoplasmic domain, improved larval oyster survival to no treatment control levels (95% survival). The WalKR regulon has been studied in *S. aureus*, and mutant strains of *S. aureus* lacking the cytoplasmic binding domain of WalK eliminate divalent cation binding and virulence of *S. aureus* (35). Here, the newly described involvement of a WalK-like protein in *P. inhibens* S4 was evaluated for its effects on quorum sensing mediated probiotic function. Our findings suggest that the lack of a cytoplasmic domain in PgaK causes various probiotic activities to be up-regulated. These findings suggest that a fully functional PgaK downregulates QS. The knockout of *pgaK* result in greater production of the C_{14} AHL and more probiotic activity such as biofilm formation, inhibitory effects against *Vibrio* and greater oyster larvae survival.

The activities of the S4Sm QS mediated probiotic suite, along with other previously described probiotic interactions, aid to further understand the full probiotic potential of this organism and demonstrate how this beneficial probiont can consistently reduce mortality in an aquaculture setting. Increased knowledge of the *P. inhibens* S4Sm probiotic genes and mechanisms involved in thwarting oyster infection should help inform efforts to prevent larval and juvenile vibriosis.

Materials & Methods

Bacterial strains, plasmids and growth conditions

P. inhibens S4Sm strains and *V. coralliilyticus* RE22Sm strains (Table 3) were routinely cultured in yeast peptone broth plus 3% NaCl (YP30), yeast peptone broth plus 3% Instant Ocean© sea salt (mYP30), or Marine Minimal Medium (3M) plus 5% sucrose (25), supplemented with the appropriate antibiotic(s) in a shaking water bath (200 RPM) at 27°C. Overnight cultures of *P. inhibens* S4Sm or *V. coralliilyticus* RE22Sm, grown in mYP30, were harvested by centrifugation (8,000 × *g*; 10 min; 4˚C), and the pelleted cells washed twice with sterile Nine Salt Solution (NSS) (36). Washed cells were resuspended to the appropriate cell densities in experimental media. *E. coli* strains were routinely cultured in LB20 (37). Antibiotics were used at the following concentrations: streptomycin, 200 µg/ml (Sm^{200}); chloramphenicol, 5 µg/ml (Cm^{5}) for *V. coralliilyticus* and *P. inhibens*, and chloramphenicol, 20 µg/ml (Cm^{20}) for *E. coli*; kanamycin, 50 µg/ml (Km^{50}) for *E. coli*, kanamycin, 80 µg/ml (Km^{80}) for *V. coralliilyticus* and *P. inhibens* grown in liquid media, and kanamycin 80 µg/ml (Km^{80}) for *V. coralliilyticus* or *P. inhibens* grown on solid media. Agar plates were prepared using Difco Bacto© agar at 1.6%.

LCMS/MS AHL quantification

P. inhibens S4Sm wild-type and mutant strains were grown under standard culture conditions as described above (mYP30, shaking at 27°C). Cultures were centrifuged at 2500 rpm for 10 min and the supernatant was desalted using a 30 mg/3 mL Strata™-X 33 µm polymeric solid phase extraction tube (Phenomenex). Bacterial supernatant (8.0 mL) was loaded onto a preconditioned column (6.0 mL of methanol followed by 6.0

mL of H_2O), then washed with 6.0 mL of 10% methanol in H_2O. Next, AHLs were eluted with 1.0 mL of methanol + 0.1% formic acid (FA), concentrated *in vacuo*, and reconstituted at 0.25 mg/mL in 50:50 methanol/water for LCMS/MS analysis. Production of *N*-acyl homoserine lactones (22) was analyzed by LCMS/MS using multiple reaction monitoring (MRM) whereby molecular ions and the neutral loss of 101 Da, corresponding to 2-amino-gamma butyrlactone, were detected (38). LCMS/MS was accomplished using an AB Sciex QTrap 4500 coupled to a Shimadzu Prominence UFLC system with the following LC conditions: Kinetex® 2.6 µm C8 100 Å 150 × 2.1 mm column (Phenomenex) at 40°C; flow rate of 0.200 mL/min; mobile phase A was 0.1% FA in H_2O; mobile phase B was 0.1% FA in methanol; a linear gradient of 50% to 100% mobile phase B occurred over 4 min and was then held at 100% B for 14 min. Mass spectrometry was conducted between 3.5 - 9.0 min in ESI positive ionization mode with the following parameters: spray voltage 5.5 kV, nebulizer gas 30, curtain gas 25, ion spray temperature of 350°C. MS/MS molecule parameters are as follows: declustering potential 96.0, entrance potential 10.0, collision energy 41.0, collision cell exit potential 9.0.

Growth rate determination

Cultures of *P. inhibens* S4Sm WT and QS mutants were grown for 48 h at 27 ^oC shaking at 200RPM and back diluted 1:1000 in mYP30 broth containing appropriate antibiotics, shaking at 200 RPM at 27 ^oC in a volume of 10ml mYP30 in 125 ml borosilicate bottles. Viable cell density (CFU/ml) was determined by serial dilution and spot plating at 2 h intervals, and OD_{600} was quantified by spectrophotometry at 600 nm.

Allelic exchange mutagenesis

The modified pDM4 plasmid containing a kanamycin resistance (Km^R) gene, pDM5, was used to construct the allelic exchange mutants (Table 3) as described by Gibson *et al.* (39). The Km resistance gene was amplified from the TOPO2.1 vector (Invitrogen) and inserted into pDM5 via the Gibson Assembly Reaction at the AgeI restriction site. pDM5 was linearized at the SacI restriction enzyme site, using SacI-HF (New England Biolabs), within the multicloning region (MCR) for all mutation destined Gibson Assemblies. The ligation mixture was introduced into *E. coli* Sm10 (containing λpir) by electroporation with the BioRad Gene Pulser II in a 2 mm cuvette (2.5 kV; 25 µF; 200 Ω). Transformants were selected by growth on $LB20Cm_{20}$ agar plates, and successful mutagenesis was confirmed by PCR screening for a novel junction between the pDM5 plasmid and the Gibson Fragment(s) from *P. inhibens*. The mobilizable suicide vector was transferred from *E. coli* Sm10 into *P. inhibens* S4Sm by conjugation as previously described (40). Transconjugants were selected by utilizing the kanamycin resistance (Km^R) gene located on the suicide plasmid. The subsequent incorporation of the target gene fragments into the suicide vector was confirmed by PCR analysis using specific primers (Table 4) to screen for the novel genetic inserts into the plasmid. The double crossover transconjugants were selected for by growth on $3MSm^{200}$ +5% sucrose agar plates for a second crossover event. Sucrose is used as the counter selective agent because pDM5 contains the *sacB* gene, which encodes levansucrase that converts sucrose to toxic levan (41). Putative allelic exchange mutants were screened for kanamycin sensitivity. The resulting S4Sm

mutants were then screened for the desired allelic exchange double crossover using PCR amplification.

Biofilm Formation Assay

Biofilm formation was assessed using a modification to the crystal violet (CV) staining method (42). Here, bacterial strains were grown for 24 h in mYP30Sm^{200} (27°C with shaking; 200 RPM) and were diluted to ~1×10^4 CFU/ml in 5 ml of fresh mYP30Sm^{200} containing one sterile coverslip per well in a sterile 6-well tissue culture dish (untreated polystyrene) and were allowed to grow without shaking at 27 ^{o}C for 24h. Supernatant cell densities (CFU/ml) were quantified by serial dilution and spot plating on mYP30Sm^{200}. Biofilm cell density (CFU/coverslip) was quantified by removing the coverslip and washing twice in 10 ml NSS for 5 minutes. Next, the coverslip was transferred to a 50 ml conical tube containing 10 ml NSS and 0.5 g sterile glass beads (100 µm), and vortexed vigorously for 60 seconds.

Zone of inhibition assays

The bacteriostatic/bactericidal action of TDA produced by S4Sm QS mutants against *V. coralliilyticus* RE22 and *V. anguillarum* NB10. Briefly, *Vibrio* spp. will be grown under standard conditions and diluted and spread plated at ~10^4 CFU/mL – this density will result in a nearly confluent lawn of bacteria. Then 10 µl spots of 48h S4Sm QS mutant cultures will be spotted onto the lawn of *Vibrio*. Zones of inhibition will be measured from the edge of the S4Sm colony to the outer edge of the zone of clearing to measure the zone of inhibition by TDA.

Bacterial killing assays

Assays for determination of T6SS-mediated killing were carried out as described by Salomon *et al.* (43). Briefly, an attacker-to-prey ratio of 4:1 (MOI of 4), based on CFU/ml, was used. A mixture of attacker and prey cells was filtered onto a 0.22 μm filter and placed on appropriate solid growth media for 4 h. The filter was then removed from the agar plate and vortexed for 1 minute in 10 ml NSS, the culture supernatant serially diluted, and plated on appropriate differential media to enumerate the attacker cells and remaining prey cells. TCBS agar was used to select for *Vibrio* spp. and mYP30 agar containing selective antibiotics was used to select for *Phaeobacter inhibens*.

Larval oyster experimental challenges

Assays for to determine the protective capabilities of *P. inhibens* S4Sm wild type and mutant strains against *V. coralliilyticus* mortality against eastern oyster larvae were performed as previously described by Zhao *et al.* (2016) with minor modifications. Larval eastern oysters (*Crassostrea virginica*) (6 to 10 days of age, 50 – 150 μm in size) were obtained from the Blount Shellfish Hatchery at Roger William University (Bristol, RI, USA), Virginia Institute of Marine Science (Gloucester Point, VA, USA) or Niantic Bay Shellfish Farm (Niantic, CT, USA), and were allowed to acclimate for 24 h at room temperature with gentle rocking. Next, ~100 oysters were placed in each well of a 6 well plate containing 5 ml of sterilized filtered artificial seawater at 2.8% salinity. For protection experiments by *P. inhibens* S4Sm and mutants, were added to select wells at a final concentration of 10^4 CFU/ml and allowed to colonize for 24 h before introduction of the pathogen. Next, the pathogen,

V. coralliilyticus RE22Sm (wild type or mutant strain was added to the challenge wells at a final concentration of 10^5 CFU/ml and incubated for 24 h. Larval oysters were fed with commercial algal paste (20,000 cells/ml); Reed Mariculture Inc., San Jose, CA, USA) in order to promote ingestion of the probiotics. Control wells will include non-treated larvae (with and without pathogen) and larvae incubated with probiotics but not with the pathogen. Each treatment was run in triplicate and each experiment was done at least two times. Larval survival was determined 20-26 h after addition of the pathogen.

The survival rate is calculated using the formula:

Survival rate (%) = 100 x (live larvae/total number of larvae)

Statistical analysis

Two-tailed Student's *t* tests assuming unequal variance were used for all statistical analyses for all detailed experiments. *P* values of < 0.05 were considered to be statistically significant.

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CS & DRN designed the study. CS created the mutant strains used in this study. CS performed all experiments under the supervision of DRN. CS and DRN wrote the manuscript with contributions and edits from DCR and MGC. Manuscript formatting was performed by CS and DRN. All authors read and approved the final version of this manuscript.

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Table 1. Bacterial strains and plasmids used in this study.

Strain	**Description**	**Resistance**	**Reference**
P. inhibens			
S4	Formerly named *Phaeobacter gallaeciensis* strain S4; wild-type isolate from inner shell of healthy oyster		Karim *et al* 2013
S4Sm	Spontaneous Sm^r mutant of S4	Sm^r	Zhao *et al* 2016
S4SmKm	Sm^r Km^r; mutant of S4Sm harboring the empty shuttle vector pSUP203	Sm^r Km^r	This study
S4Sm Δ*pgaI*	Sm^r Km^r; Allelic exchange mutant deficient in *luxI* homologue AHL synthase	Smr Km^r	This study
S4Sm Δ*pgaR*	Sm^r Km^r; Allelic exchange mutant deficient in *luxR* homologue AHL receptor	Sm^r Km^r	This study

S4Sm Δ*luxO*	Sm^r Km^r; Allelic exchange mutant deficient in *luxO* homologue phosphotransfer protein for QS signal transduction	Sm^r Km^r	This study
S4Sm Δ*pgaK*	Sm^r Km^r; Allelic exchange mutant deficient in *pgaK* dual function histidine kinase/phosphatase AHL receptor	Sm^r Km^r	This study
S4Sm WZ10	*clpX* insertional mutant of S4Sm	Sm^r Cm^r	Zhao *et al* 2016
S4Sm WZ20	*exoP* insertional mutant of S4Sm	Sm^r Cm^r	Zhao *et al* 2016
S4Sm WZ30	*tdbD* insertional mutant of S4Sm	Sm^r Cm^r	This study
V. coralliilyticus			
RE22	Wild-type isolate from oyster larvae		Estes *et al* 2004
RE22Sm	Spontaneous Sm^r mutant of RE22	Sm^r	Zhao *et al* 2016
V. anguillarum			

NB10	Wild type, serotype O1, clinical isolate from the Gulf of Bothnia		Norqvist *et al* 1989
NB10Sm	Spontaneous Sm^r mutant of NB10	Sm^r	Zhao *et al* 2016
E. coli			
Sm10	*Thi thr leu tonA lacY supE recA* RP4-2 Tc::Mu::Km (λ)	Km^r	Simon *et al* 1983
Sm99	Sm10 harboring pDM4	Cm^r	This study
Sm100	Sm10 harboring pDM5	Cm^r Km^r	This study
CS201	Sm10 harboring pDM5-*pgaI*	Cm^r Km^r	This study
CS202	Sm10 harboring pDM5-*pgaR*	Cm^r Km^r	This study
CS203	Sm10 harboring pDM5-*luxO*	Cm^r Km^r	This study
CS204	Sm10 harboring pDM5-*pgaK*	Cm^r Km^r	This study
Plasmids			
pDM4	Cm^r; suicide vector with R6K origin and	Cm^r	Milton *et al* 1996

	sacB		
pDM5	Cm^r Km^r; suicide vector with R6K origin and *sacB*	Cm^r Km^r	Schuttert *et al* 2021
pSUP202P	Ap^r Cm^r Tc^r; broad host shuttle vector	Ap^r Cm^r Tc^r	Simon *et al* 1983
pSUP203	Ap^r Cm^r Tc^r Km^r; broad host shuttle vector	Ap^r Cm^r Tc^r Km^r	Schuttert *et al* 2021

Table 2. Primers used in this study

Primer	**Sequence (5' to 3', underlined sequences are engineered for Gibson Assembly sites in pDM5)**	**Description**
PmI23	tgtggaatcccgggagagctAACGAACGATCAAACCAC	For *pgaI* insertional mutation, 5' forward
PmI24	tgaccggaggCCGATCGCTCGGTTTTAG	For *pgaI* insertional mutation, 5' reverse
PmI25	gagcgatcggCCTCCGGTCAGCTCAGGA	For *pgaI* insertional mutation, 3' forward
PmI26	gcatgcgggtaacctgagctAAGGAGAAGAGCGCGACG	For *pgaI* insertional mutation, 3' reverse
PmR19	tgtggaatcccgggagagctATTTTATGGCAACCAAAGTTAATC	For *pgaR* insertional mutation, 5' forward
PmR20	ttatggctgaAAAGTGATAGTGATCGCC	For *pgaR* insertional

		mutation, 5’ reverse
PmR21	ctatcacttttTCAGCCATAACTGCAACG	For *pgaR* insertional mutation, 3’ forward
PmR22	gcatgcgggtaacctgagctATAGCATGCGTGGTGTTG	For *pgaR* insertional mutation, 3’ reverse
PmO27	tgtggaatcccgggagagctAGAACATGCATGATGTGGC	For *luxO* insertional mutation, 5’ forward
PmO28	tgtcggaccgCCACCGGATCAATGTGGTG	For *luxO* insertional mutation, 5’ reverse
PmO29	gatccggtggCGGTCCGACAGCGAATGC	For *luxO* insertional mutation, 3’ forward
PmO30	gcatgcgggtaacctgagctATTTCGTGCTGAAACCGTTTCG	For *luxO* insertional mutation, 3’

		reverse
PmK15	tgtggaatcccgggagagctATTGCGGATAATGCCCCTTATAAG	For *pgaK* insertional mutation, 5’ forward
PmK16	ctgaggatcaAGACCGGCGAATTGAAAG	For *pgaK* insertional mutation, 5’ reverse
PmK17	tcgccggtctTGATCCTCAGCGAGGTGG	For *pgaK* insertional mutation, 3’ forward
PmK18	gcatgcgggtaacctgagctAAAGATCTGATCCTGCTTCTCC	For *pgaK* insertional mutation, 3’ reverse

Figure 1. LCMS/MS detection of AHLs produced by *P. inhibens* S4Sm WT and QS mutants. (A) Multiple reaction monitoring (MRM) chromatogram showing the production of (3*R*)-*N*-(3-hydroxydecanoyl)-L-homoserine lactone production by *P. inhibens* S4Sm WT and QS mutant strains. The molecular ion of 271.2 Da was detected along with an accompanying neutral loss of 101.0 Da. Chromatograms are shown for wild type S4Sm (blue), *pgaI* mutant (red), *pgaR* mutant (green), *luxO* mutant (light blue), and *pgaK* mutant (grey). (B) MRM chromatogram showing the production of (3*R*,7*Z*)-*N*-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone production by *P. inhibens* S4Sm WT and QS mutant strains. The molecular ion of 326.2 Da was detected along with an accompanying neutral loss of 101.0 Da. Chromatograms are shown for wild type S4Sm (blue), *pgaI* mutant (red), and *pgaR* mutant (green), *luxO* mutant (light blue), and *pgaK* mutant (grey).

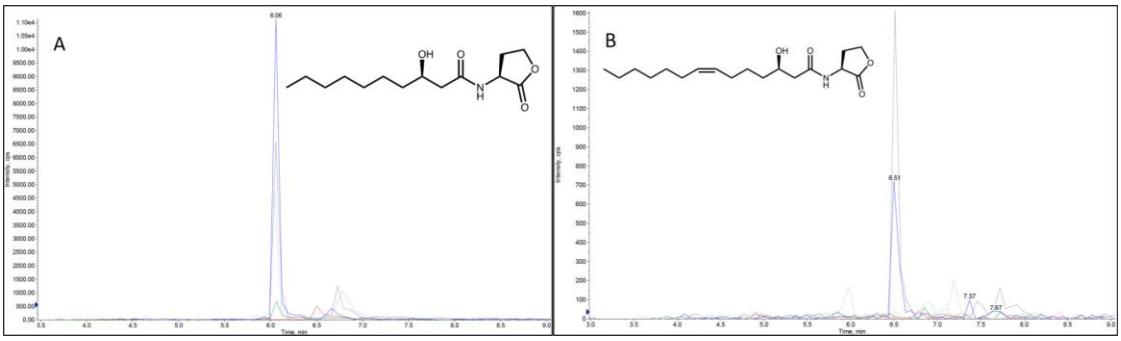

A
OH
O
N
H
O
O
6.06
Intensity, cps
Time, min
B
OH
O
N
H
O
O
6.51
7.37
7.67
Intensity, cps
Time, min

Figure 2. Growth of *P. inhibens* S4Sm wild type and QS mutant strains during growth at 27°C under shaking conditions. Determination of *P. inhibens* S4Sm wild type and QS mutant viable cell growth curves over 24 h at 27°C. Cell density (CFU/ml) quantification determined by serial dilution and spot plating in triplicate. The data are the average of three biological replicates (experiments); each experiment included three technical replicates. Error bars represent ±1 standard deviation (SD). Statistical analysis by Student's T-test and compared to *P. inhibens* S4Sm WT. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$.

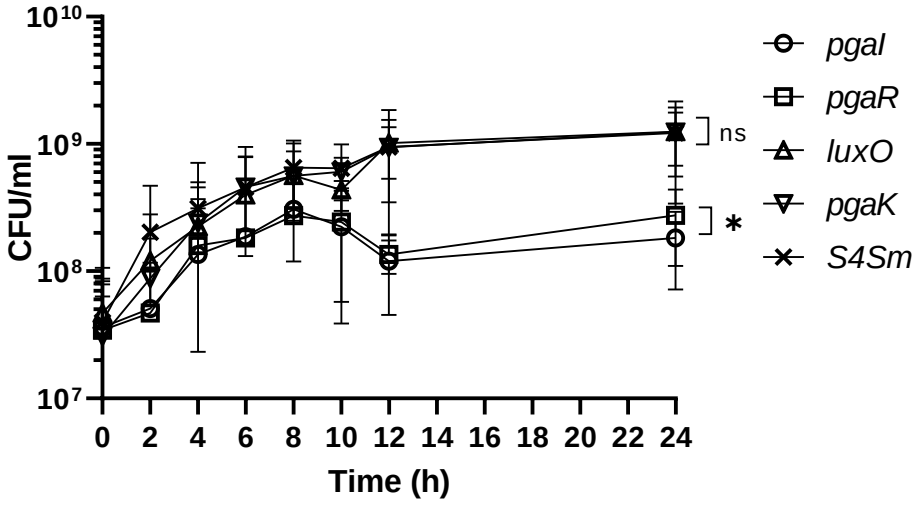

10^{10}
10^{9}
10^{8}
10^{7}
CFU/ml
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
pgaI
pgaR
luxO
pgaK
S4Sm
ns
*

Figure 3. Growth of *P. inhibens* S4Sm WT and QS mutants in static culture conditions and biofilm formation on glass coverslips, as described in the Materials & Methods. (A) Mean cell density during planktonic growth in static culture. (B) Mean cell density during coverslip biofilm formation. S4Sm wild type and *pgaI*, *pgaR*, *luxO*, and *pgaK* mutant strains were grown in 6-well tissue culture plates containing 5 ml of mYP30 for 24 h at 27°C. Planktonic growth was determined by serial dilution and spot plating. Average of 3 experiments; error bars indicate ±1 SD. Statistical comparisons were made against S4Sm WT planktonic growth, and biofilm formation values. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$. Statistical analysis by unpaired Student's T-test.

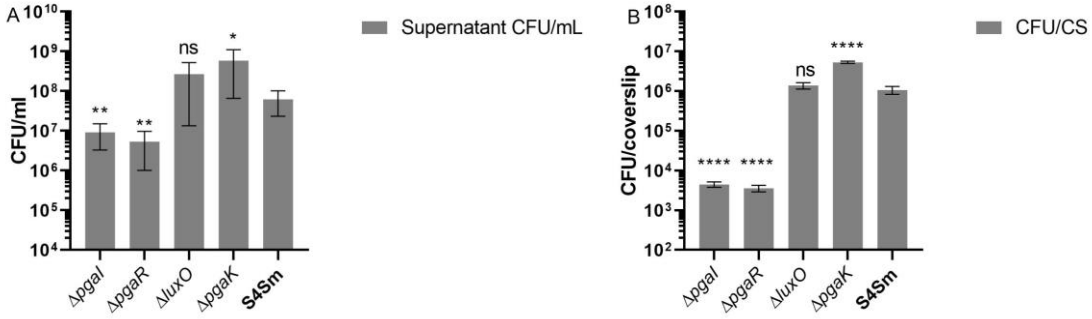

A
CFU/ml
Supernatant CFU/mL
**
**
ns
*
ΔpgaI
ΔpgaR
ΔluxO
ΔpgaK
S4Sm
B
CFU/coverslip
CFU/CS
****
****
ns
****
ΔpgaI
ΔpgaR
ΔluxO
ΔpgaK
S4Sm

Figure 4. Growth inhibition of *V. coralliilyticus* RE22Sm and *V. anguillarum* NB10Sm by *P. inhibens* QS mutants and S4Sm WT (A) Quantification of zone of inhibition (ZOI) ability of S4Sm WT and mutant strains against a *V. coralliilyticus* RE22Sm lawn. (B) Inhibitory effect of S4Sm WT and mutant strains against a *V. anguillarum* NB10Sm (serotype O1) lawn. Average of 3 experiments; error bars indicate ±1 SD. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$ (Statistical analysis by unpaired Student's T-test).

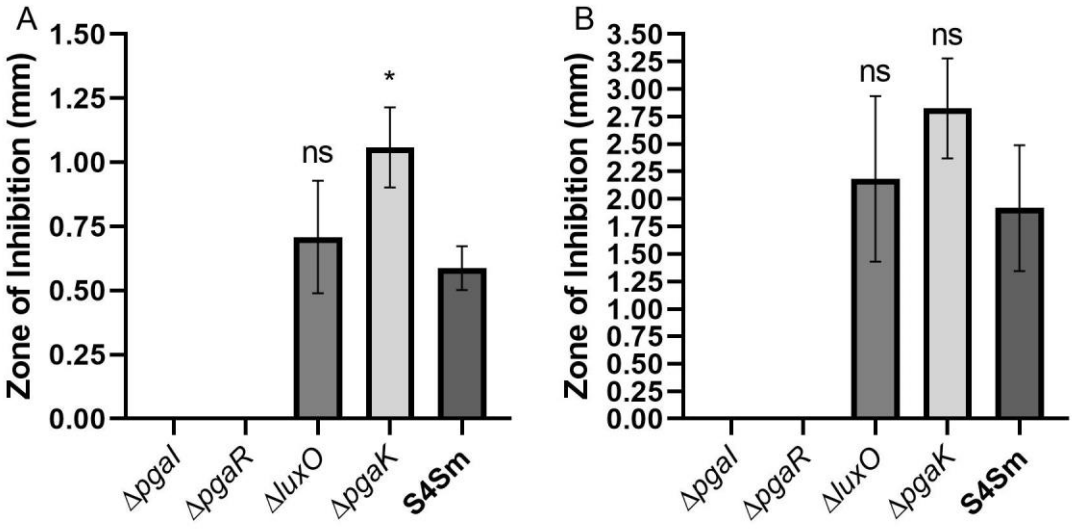

A
Zone of Inhibition (mm)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
ns
*
ΔpgaI
ΔpgaR
ΔluxO
ΔpgaK
S4Sm
B
Zone of Inhibition (mm)
3.50
3.25
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
ns
ns
ΔpgaI
ΔpgaR
ΔluxO
ΔpgaK
S4Sm

Figure 5. Determination of the *V. coralliilyticus* RE22Sm (attacking cell) T6SS-mediated antibacterial activity against *P. inhibens* S4Sm WT and QS mutants (prey cell) when incubated on a filter for 4 h at 27°C with a 4:1 predator: prey ratio (MOI = 4) as described in the Materials & Methods. Each bar shows the cell density of S4Sm cultures after 4 h incubation with *V. coralliilyticus* RE22Sm cells. Average of 3 experiments; error bars indicate ±1 SD. Statistical analysis by unpaired Student's T-test, and compared to S4Sm WT cell densities. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$.

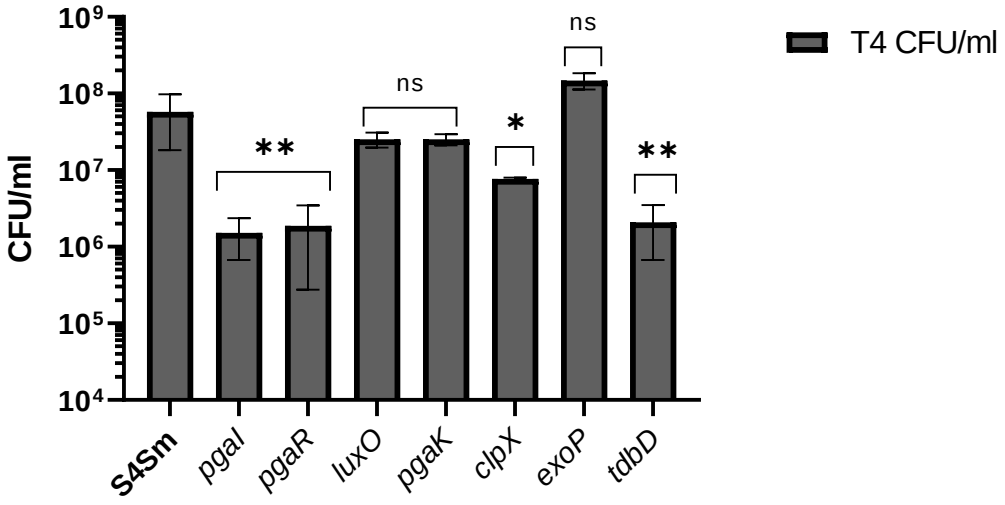
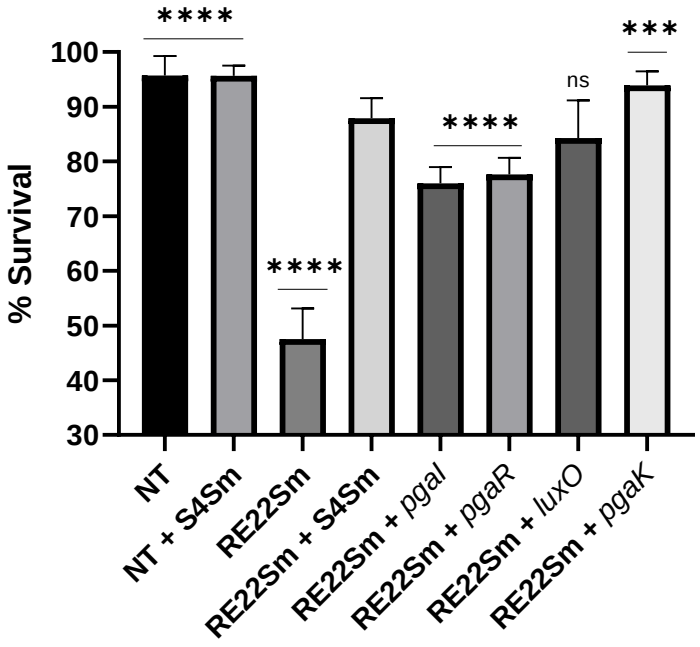

CFU/ml
10^9
10^8
10^7
10^6
10^5
10^4
**
ns
*
ns
**
S4Sm
pgaI
pgaR
luxO
pgaK
clpX
exoP
tdbD
T4 CFU/ml

Figure 6. Oyster larvae survival after pretreatment with *P. inhibens* S4Sm WT or mutant strains for 24 h (~1×10^4 CFU/ml) and then challenged with *V. coralliilyticus* RE22Sm (~1×10^5 CFU/ml). Oyster larvae treated with artificial seawater served as the negative control. Larval survival (% ±1 SD) was determined 24 h after challenge. Average of at least 3 biological replicates; error bars indicate ±1 SD; Statistical comparisons were made against RE22Sm + S4Sm challenge group. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$. Statistical analysis by unpaired Student's T-test.


****
***
ns
****
****
100
90
80
70
60
50
40
30
% Survival
NT
NT + S4Sm
RE22Sm
RE22Sm + S4Sm
RE22Sm + pgaI
RE22Sm + pgaR
RE22Sm + luxO
RE22Sm + pgaK

Appendix A (additional figure for Manuscript III)

Figure 1. Proposed interaction network between *P. inhibens* S4Sm and *V. coralliilyticus* RE22Sm